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EVALUATION AND IDENTIFICATION OF MICROBIAL CONTAMINANTS IN POLYHERBAL MEDICINES USED FOR THE TREATMENT OF TUBERCULOSIS IN AMATHOLE DISTRICT MUNICIPALITY, EASTERN CAPE PROVINCE, SOUTH AFRICA

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DEPARTMENT OF BIOCHEMISTRY AND MICROBIOLOGY

FACULTY OF SCIENCE AND AGRICULTURE

UNIVERSITY OF FORT HARE

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ELIZABETH BOSEDE FAMEWO

A thesis submitted in fulfilment of the requirements for the degree of
DOCTOR OF PHILOSOPHY IN MICROBIOLOGY

DEPARTMENT OF BIOCHEMISTRY AND MICROBIOLOGY
FACULTY OF SCIENCE AND AGRICULTURE
UNIVERSITY OF FORT HARE, ALICE,
SOUTH OF AFRICA

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MAY, 2018

DEDICATION

I dedicate this piece of work to the glory of my Lord and Saviour Jesus Christ who has blessed me beyond anything I could ever have imagined and who has loved me beyond my comprehension.

DECLARATION

I, Elizabeth Bosede Famewo, declared that this thesis, submitted to the University of Fort Hare for the degree of Doctor of Philosophy in the Department of Biochemistry and Microbiology in the Faculty of Science and Agriculture, is my own work; and that this work has not been submitted to any other institution for the award of any academic degree.

I declare that I followed the rules and conventions concerning referencing and citation in scientific writing.

I also declare that all sources of materials used for this thesis have been duly acknowledged and accurately referenced.

Again, I declare that I am fully aware of the University of Fort Hare policy on plagiarism and I have taken every precaution to comply with the regulations of the University.

Name: Elizabeth Bosede Famewo **Signature:**.....

Institution: University of Fort Hare **Date:** May, 2018

We confirm that the work reported here was carried out by the above named candidate under our supervision.

Prof Anthony Jide Afolayan

Signature: ……. Date: ……

Prof Anna Maria Clarke

Signature: ..... Date: .....

INTELLECTUAL PROPERTY RIGHTS CONSIDERATIONS

The ethno-medicinal survey conducted for this thesis was carried out with the full consent of all participants, with further verbal agreement and understanding that this research shall not be used for commercial purposes, but shall serve as enlightenment on the safety and efficacy of the polyherbal medicines. It would also serve as a means of preservation of the indigenous knowledge of polyherbal medicines used for the treatment of tubeculosis in the Eastern Cape Province, South Africa.

ETHICAL APPROVAL FOR THE STUDY

The portion of this study involving the ethno-medicinal survey on the medicinal plants used for the preparation of polyherbal medicines used for treatment of tuberculosis was carried out following the approval of the University of Fort Hare's Ethics Committee, number AFO061SFAM01.

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TABLE OF CONTENTS

Contents **Page No**

GENERAL ABSTRACT

General Abstract

Tuberculosis is caused by *Mycobacterium tuberculosis*. The emergence of drug-resistant strains of this organism has become a global public health problem. In the continuing search for effective treatment, polyherbal medicines offer a great hope in the development of alternative drugs for the treatment of tuberculosis. The use of herbal formulations for therapeutic purposes has significantly increased in the developed and developing countries because of their curative property, less toxicity and minimal side effects. However, there is little information on their safety and effectiveness in the literature. To address this, polyherbal medicines used for the treatment of tuberculosis in the Eastern Cape Province of South Africa were evaluated.

Ethno-medicinal survey was conducted through semi-structured questionnaires coupled with informal conversations with the herbal sellers in five communities in the study area. Bacterial and fungal DNA was extracted from the polyherbal medicines purchased. A fragment of the bacterial 16S rRNA gene and internal transcribed spacer region of the fungal rRNA operon were amplified with universal primers 27F and 518R, and ITS1 and ITS4 respectively. Following standard procedures, the amplicons were finally run on Illumina's MiSeq platform. Furthermore, the remedies were screened against *Mycobacterium tuberculosis* H37Rv using Middlebrook 7H9 media and MGIT BACTEC 960 system. Agar dilution method was used to determine the minimum inhibitory concentration (MIC) of the remedies against eight bacteria and three fungi isolates. The herbal preparations were assayed for their toxicity using hatchability success and larval mortality of *Artemia salina* Leach. Finally, their nutritive properties were analysed using an inductively coupled plasma optical emission spectrometer for mineral analysis while the vitamins were determined using standardized methods.

A total of nine polyherbal preparations were collected. The herbs used for the preparation of these remedies belong to 20 families. Apiaceae [5(25%)] was the most prominent plant family used, followed by Liliaceae [4(20%)], Strychnaceae [4(20%)], Rutaceae [4(20%)] and Hypoxidaceae [3(15%)]. The two most frequently used plants were *Allium sativum* L. (Liliaceae) and *Strychnos decussata* (Pappe) Gilg. *(*Strychnaceae). Rhizomes was the most common parts used, followed by the roots and barks. The herbal medicines were prepared mainly by infusion and decoction.

The presences of pathogenic and non-pathogenic bacteria were identified in the polyherbal medicines. Generally, the most common bacteria identified from the samples were *Bacillus* sp., *Enterobacter* sp., *Klebsiella* sp., *Rahnella* sp., *Paenibacillus* sp., *Clostridium* sp. and *Pantoea* sp. The predominant mycoflora obtained belongs to different genera or species of fungi; these include *Alternaria*, *Candida*, *Ramularia*, *Cladosporium*, *Penicillium*, *Aspergillus* and *Malassezia*.

The susceptibility testing revealed that all the remedies contain anti-tubercular activity against *M. tuberculosis* H37Rv at concentrations below 50 ug/ml. Seven of the polyherbal preparations showed activity at concentrations below 25 ug/ml. The MIC values exhibited inhibitory activity at 1.562 µg/ml. However, isoniazid showed more inhibitory activity against *M. tuberculosis* at 0.05 µg/ml when compared to the polyherbal remedies.

The inhibitory activity of the polyherbal medicines based on the overall MIC revealed that Hogsback first site (HBfs) and Fort Beaufort (FB) remedies were the most active remedies against the bacterial isolates at the concentration of 2.5 mg/mL. Among the nine herbal formulations, only King Williams Town site A (KWTa) remedy showed activity against *Aspergillus niger* and *Aspergillus fumigatus* with the MIC valve of 2.5 mg/mL. While King

Williams Town site C (KWTc) and Hogsback third site (HBts) had the highest activity at 1.25 mg/mL against *Candida albicans*, the remaining remedies were active at 2.5 mg/mL.

The percentage hatchability of 44.42%, 42.96% and 39.70% were observed in *A. salina* cysts incubated with herbal preparations from KWTa, HBfs and HBts respectively. The hatching success of the cysts in these remedies was significantly higher than the positive control (nystatin) and the negative control (sea water) at $p < 0.05$. The mortality of *A. salina nauplii* incubated in Alice (AL), King Williams Town site B (KWTb) and KWTc remedies were significantly higher than when larvae were incubated in both controls. Based on Meyer's index, the LD_{50} of each polyherbal medicine was between 2.9 and 4.0 mg/ml, the LD_{50} values greater than 1 mg/ml, an indication that they are not toxic.

The polyherbal preparations were found to be rich in vitamins and mineral nutrients. Calcium was the highest macronutrient detected while the lowest nutrient was phosphorus. Iron was the highest micronutrient in the majority of the polyherbal preparations while the lowest value was recorded for copper. Vitamin C was absent in the herbal preparations while vitamin A and E were detected.

This study provides significant ethno-medicinal information on polyherbal medicines used for the treatment of TB in the study area. The presence of the identified bacteria and fungi in the herbal formulations is a cause for concern. However, the ability of the remedies to possess activity against *Mycobacterium tuberculosis* and other pathogenic microorganisms associated with tuberculosis infection makes them potential sources of new antimycobacterial agents. Also, they are rich sources of mineral nutrients, and are as well non-toxic, thus, they are safe for consumption. In view of their anti-tubercular properties, this study has provided a better understanding of the reasons why TB-patients make use of these polyherbal formulations. Also, the study supports the folkloric use of polyherbal medicines in the treatment of tuberculosis in the Eastern Cape Province, South Africa.

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

TABLE OF CONTENTS

Contents **Page No**

1.0 Introduction

1.1.0 Etiology and mode of transmission of tuberculosis

Tuberculosis (TB) is a contagious chronic bacterial infection caused by a group of closely related bacterial species called *Mycobacterium tuberculosis* complex. *M. tuberculosis* is the main cause of TB in humans while other members of the complex include *M. bovis*, *M. canetti*, *M. microti* and *M. africanum.* However, *M. microti* is not known to cause TB in humans; also infection with *M. africanum* is very rare, while *M. bovis* is the main cause of tuberculosis in other animal species (Sandhu, 2011). *Mycobacterium tuberculosis* is a rod-shaped, aerobic and small slow-growing bacterium that lives only in humans. This disease is mainly transmitted via direct exposure to Tubercle bacilli in airborne droplets from coughing or sneezing of individuals living with the active respiratory disease (Narwadiya et al., 2011). *M. tuberculosis* mainly affects the lungs thereby causing lung tuberculosis called pulmonary tuberculosis or other parts of the body thus leading to extra-pulmonary tuberculosis (Sharma and Mohan, 2004). The common symptoms of active tuberculosis include cough with sputum and blood, chest pains, poor appetite, weakness, weight loss, fever and night sweats. The immunological life cycle of TB is shown in Figure 1.1.

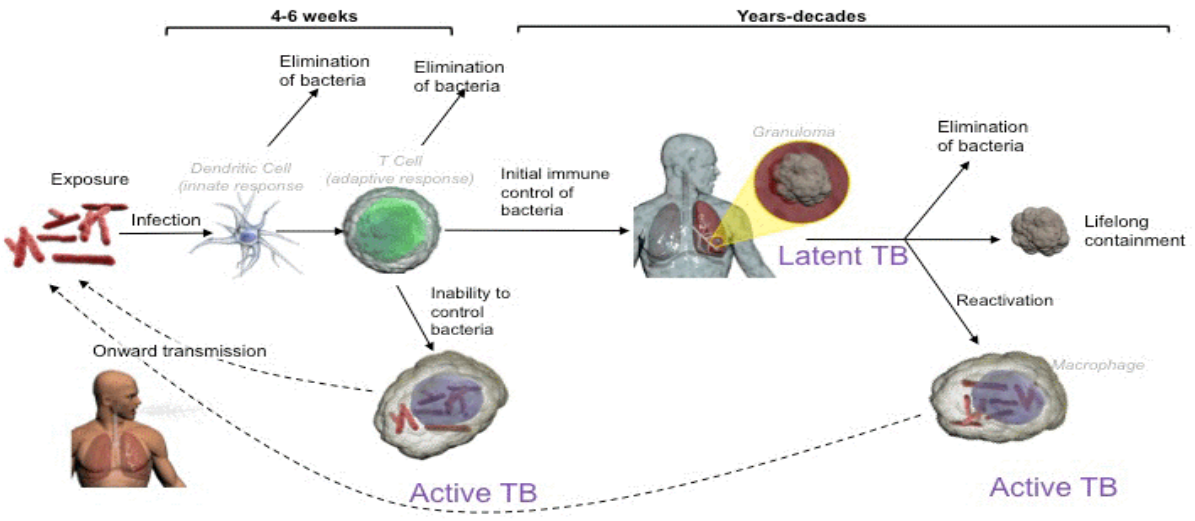

Figure 1.1: Stages in the immunological life cycle of tuberculosis (Michael, 2013)

1.1.1 Current status of tuberculosis

Tuberculosis is a worldwide pandemic and the leading cause of mortality among people infected with human immunodeficiency virus (HIV) in the developing countries. TB has been reported to be among the top ten causes of death worldwide, ranking above HIV/AIDS as the leading cause of death among the infectious diseases (WHO, 2016). It has become a major public health concern globally with over 2 billion people infected, 10.4 million cases, and 1.8 million deaths in 2015 (Baldwin et al., 2015). The largest number of new TB cases occurred in four Asian countries including India, Indonesia, China and Pakistan with 61% of new cases. This was followed by two African countries namely Nigeria and South Africa, with 26% of new cases (WHO, 2016). One-third of the world's population have been infected with latent tuberculosis; the resurgence of this disease has been attributed to the rise in HIV cases with 13% of mortalities amongst acquired immune deficiency syndrome (AIDS) patients (WHO, 2004; Anyangwe et al., 2006). In some countries in sub-Saharan Africa, about 75% of TB patients have been found to be HIV positive; and over 95% prevalence of TB deaths occurred in developing countries (Van Lettow et al., 2003)

South Africa is one of the six countries with the highest burden of TB; and the incidence rate has increased by 400% over the past 15 years most especially among people living with HIV (Lall and Meyer, 1999; Green et al., 2010). It was estimated that about 80% of South African population is infected with *M. tuberculosis* with 88% prevalence of latent TB among 30-39 years old (TBFACTS, 2015). TB has continued to be the leading cause of death in South Africa (Statistics South Africa, 2013). The WHO statistics gave an estimated incidence of 500,000 cases of active TB in 2011. This implies that about 1% of 50 million South Africans contacted active TB diseases each year (Annual Performance Plan, 2012). Out of these cases, WHO estimated that about 330,000 (66%) people have both HIV and TB infection; but the

South African Department of Health revealed that 73% of TB patients are HIV positive (Annual Performance Plan, 2012).

1.1.2 Treatment of tuberculosis and mechanism of action of TB-drugs

Tuberculosis is a curable and preventable disease. The current treatment regimens comprised the combination of at least four drugs over a period of six months. These drugs include isoniazid, rifampicin, pyrazinamide and ethambutol (Duncan and Sacchettini, 2000; Zhang and Yew, 2009). In order to achieve effective cure rate, WHO recommended 2 months of intensive phase of daily administration of isoniazid, rifampicin, pyrazinamide and ethambutol. This is followed by 4 months continuous phase of daily use isoniazid and rifampicin (WHO, 2010). Based on mechanism of action as shown in Figure 1.2, tuberculosis drugs can be classified as inhibitors of bacterial protein synthesis (aminoglycosides), electron transport across the bacterial membrane (a proposed mechanism of action for pyrazinamide), nucleic acid synthesis (rifampin, quinolones) and cell wall synthesis (isoniazid, ethambutol, ethionamide and cycloserine) (Ma et al., 2007).

Isoniazid (INH) is the most widely used treatment for TB and its latent infections (Heym et al., 1999). This drug enters the cell as a pro-drug, which is activated by *Mycobacterium tuberculosis* catalase-peroxidase enzyme (KatG). This enzyme activates INH and facilitates its interaction with various toxic reactive species (oxides, hydroxyl radicals and organic moieties) in the bacterial cell (Barry et al., 1998). Thus, weaken the components of the cell wall and finally, causing the death of the bacteria. INH targets *inhA* enzyme (enoylacyl carrier protein reductase), which is involved in the elongation of fatty acids in mycolic acid synthesis (Zhang and Telenti, 2000). The replacement of an amino acid in the NADH binding site of *inhA* results into INH resistance, preventing the inhibition of mycolic acid biosynthesis (Telenti et al., 1993). INH-resistant strains often lose catalase and peroxidase activities due to

KatG Ser315Thr mutation (Hazbon et al., 2006). Resistance to INH can also occur through mutations in the promoter region of *inhA*. This leads to over expression of *inhA*, or by mutations at the *inhA* active site, thereby lowering *inhA* affinity for INH (Rozwarski et al., 1998).

Rifampicin (RIF) has been used as a first-line drug in combination with other therapies for the treatment of TB infections. RIF is believed to inhibit bacterial DNA-dependent RNA polymerase (Shehzad et al., 2013). This drug interferes with RNA synthesis by binding to the β subunit of mycobacterial RNA polymerase, which is encoded by *rpo*B, thereby killing the organism. Resistance to RIF arises due to missense mutations in the gene. *M. tuberculosis* resistance to RIF occurs at a frequency of 10^{-7} to 10^{-8} as a result of mutations in *rpo*B (Dye and Williams, 2010). About 96% of all mutations are found in the 81-bp core region of the gene between codons 507 and 533, with the most common changes occurring in codons Ser531Leu, His526Tyr and Asp516Val (Zhang et al., 2003).

Pyrazinamide (PZA) is another vital first-line drug used for the treatment of tuberculosis. It played an important role in reducing the duration of TB treatment (Salfinger et al., 1990). PZA is a pro-drug that requires conversion to its active form, pyrazinoic acid (POA) by the mycobacterial enzyme pyrazinamidase/nicotinamidase. The efflux system of the mycobacterial cell enables massive accumulation of POA in the bacterial cytoplasm, leading to disruption of the bacterial membrane potential (Zhang et al., 1999; Sheen et al., 2009). The exact mechanism of PZA resistance remains unknown. However, PZA resistance has been associated with defective pyrazinamidase/nicotinamidase activity which results from mutations that might occur at different regions (3-17, 61-85 and 132-142) of pyrazinamidase/nicotinamidase (Scorpio and Zhang, 1996).

Ethambutol (EMB) is a first-line drug used in combination with INH, RIF and PZA preventing the emergence of drug resistance mycobacterium. This drug interferes with the cell wall of *M. tuberculosis* through a synthetic mechanism thereby inhibiting arabinosyl-transferase (*embB*), an enzyme involved in cell wall biosynthesis (Telenti et al., 1997). The enzyme has been proposed as the target of EMB in *M. tuberculosis* (Zhang and Yew, 2009). Mutation is the cause of ethambutol resistance and it occurs at a rate of approximately 1 in 10^7 organisms. It increases the production of arabinosyl-transferase, which overwhelms the inhibitory effects of ethambutol. Studies have revealed five mutations in codon 306 accounting for 70–90% of all ethambutol resistant strains (Van Niekerk and Ginsberg, 2009).

Thus, the resistance of *M. tuberculosis* to TB-drugs is mostly due to mutation and this is a cause for concern. Therefore, it is important to search for new anti-tuberculosis agents, preferably those that can be readily and simply produced from medicinal plants.

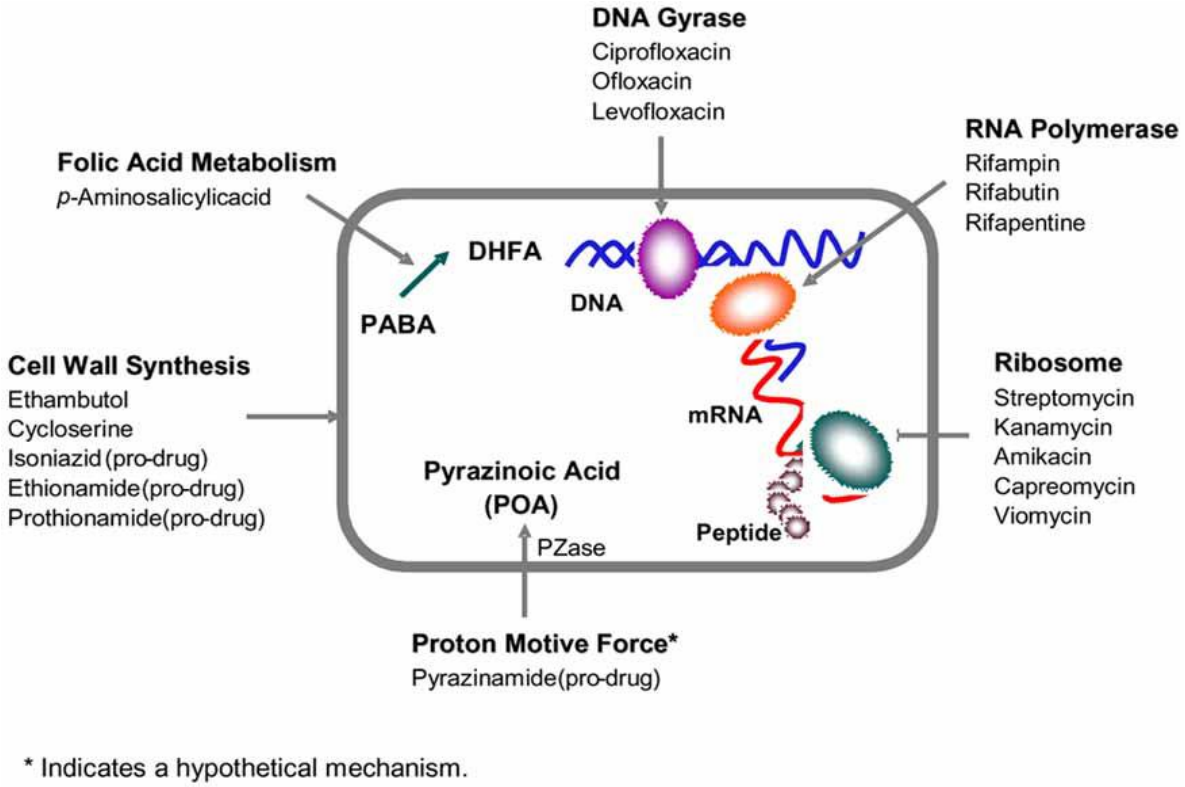

Figure 1.2: Schematic illustration of the sites of action for the available anti-tuberculosis drugs (Ma et al., 2007).

Due to the toxicity of tuberculosis medicines, they could cause serious adverse effects in the patients (Table 1.1).

Table 1.1: Adverse effects associated with anti-tubercular drugs

TB therapy	**Adverse effects**
Isoniazid	Hepatitis, skin rash
Rifampicin	Abdominal pain, nausea, vomiting, hepatitis, thrombocytopenia purpura
Cycloserine	Convulsions, dizziness, headache, depression, psychotic reactions
Pyrazinamide	Arthralgia, hepatitis
Streptomycin	Vestibular and auditory nerve damage, renal damage
Thioacetazone	Skin rash, exfoliative dermatitis
Kanamycin	Vertigo, auditory nerve damage, nephrotoxicity
Ethionamide	Diarrhoea, abdominal pain, hepatotoxicity
Ethambutol	Eye problems, retrobulbar neuritis
Paraaminosalicylic acid	Anorexia, nausea, vomiting, hypersensitivity reactions

Source: Mehta et al., 2015.

1.1.3 Challenges in the treatment of tuberculosis

Mycobacterium tuberculosis adopts diverse strategies to survive in the host lesions. These survival mechanisms make the pathogen resistant to the available TB-drugs, thus, the difficulty experienced in controlling the spread of this disease (Shehzad et al., 2013). The global epidemic of tuberculosis is further exacerbated by the existence of multi-drug resistant-TB (MDR-TB), extremely drug resistant-TB (XDR-TB) and totally drug resistant-TB (TDR) which are successively more difficult to treat. MDR-TB is the resistance of TB to

isoniazid and rifampicin, the two most powerful first-line anti-TB drugs. XDR-TB is resistant to MDR-TB drugs, all fluoroquinolones and at least one of the second-line anti-TB injectable drugs including amikacin, kanamycin and/or capreomycin (Zhang and Yew, 2009; Nguta et al., 2015). The emergence of drug resistance in a patient could occur as a result of low adherence to treatment, inadequacy of the drug regimen (such as, wrong antibiotic choices or dosages, poor drug quality) and patient-dependent pharmacodynamic and pharmacokinetic properties of the drugs administered (Pasipanodya and Gumbo, 2011).

The annual global burden of MDR-TB was estimated to 480,000 cases, 170,000 deaths, with about 9% of them being affected by XDR-TB (WHO, 2016). Treatment of MDR-TB required the use of the second-line drug up to 2 years. This process is costly and could produce severe adverse drug reactions in patients, thus only a few of the patients who entered into treatment successfully completed the treatment. Treatment failure could as well occur in some of these patients due to weaknesses in current regimens, national programmes, and operational challenges (Wallis, 2016). About 424,000 MDR TB cases are reported yearly in which 9.6% of MDR TB cases had XDR TB (WHO, 2009a; WHO, 2016). In many developing countries, the chances of recovery of patients with XDR-TB are extremely low and the spread of this strain raises the possibility of the return to a pre-antibiotic era (Raviglione, 2006; Mohajan, 2015). This type of TB is of great concern among the people infected with HIV infection. Several cases of XDR-TB have been detected in at least 55 countries (Mohajan, 2015) around the world of which South Africa is included. The WHO estimated that 40,000 cases of XDR-TB occur each year (WHO, 2009a). Due to the complexity of this TB, the patients need to be put on longer MDR-TB regimens to which one of the new drugs (bedquiline and delamanid) may be added (WHO, 2016). Another hurdle faced in the treatment of tuberculosis is the high prevalence of co-infection with *Mycobacterium tuberculosis* and HIV. These two infections are synergistic. About half of the people living with HIV/AIDS develop active TB (Corbett et

al., 2003). This disease is the leading killer of HIV positive people, with about 35% deaths in 2015 (WHO, 2016). The annual tuberculosis report of the WHO states that "without new tuberculosis drugs and regimens, it will be very difficult to improve treatment outcomes in the near future" (Anderson et al., 2015).

1.1.4 Herbal medicines

Medicinal plants have continued to play an important role in the development of potent therapeutic agents due to their numerous chemical diversities (Gautam et al., 2007). Many conventional drugs such as quinine from cinchona tree, aspirin from willow tree, codeine and morphine from *Papaver somniferum* originated from plant. Others include digoxin from foxglove, atropine, ephedrine, reserpine and artemisinin derivatives from *Artemisia annua* (Chin et al., 2006; Kurokawa et al., 2010). According to WHO, about 70-80% of the developing countries still rely on herbal medicines for their primary health care; however this varies from countries to countries (WHO, 2002a). This is because of the general belief that herbal medicines are without any side effects, readily accessible and cost effective as well as their cultural acceptability and better compatibility with the human body (Gupta and Raina, 1998; Pal and Shukla, 2003; Gratus et al., 2009; Hasan et al., 2009). Thus, the use of herbal remedies exceeds that of the conventional drugs by two to three times (WHO, 1998; Pal and Shukla, 2003).

In the developed countries, such as the United States, about 38% of adults and 12% of children make use of complementary and alternative medicine (CAM) (NCCIH, 2016). In Europe, North America and other industrialized regions, over 50% of the population have used CAM at least once. About 75% of people living with HIV/AIDS use traditional medicine (TM)/CAM in San Francisco and London; 40% of the population in Hong Kong (WHO, 2002b; Chan, 2003) and 70% of all medical doctors in France and German regularly

prescribe herbal medicine (Murray and Pizzorno, 2000). All these people used CAM for the treatment of illnessess such as cardiovascular diseases, diabetes, hypertension and depression. The use of traditional medicine is not limited to developed countries; many people in developing countries still rely on herbal medicines for their primary care (Wachtel-Galor and Benzie, 2011). In Asia and Latin America, the populations continue to use herbal medicine as a result of historical circumstances and cultural beliefs. In China, TM accounts for around 40% of all health care delivered and more than 90% of general hospitals in China have units for traditional medicine. Up to 70% of Indian and 80-90% of the population in Africa depend on traditional medicine to help meet their health care needs (WHO, 2009b). In Ghana, Mali, Nigeria and Zambia, the first line of treatment for 60% of children with high fever resulting from malaria is the use of herbal medicines (Peltzer, 2009). In South Africa, it was estimated that about three million people use indigenous medicines for their primary health care purposes, and exploration in the treatment of various ailments (Louw et al., 2002; Buwa and Afolayan, 2009). In the Eastern Cape Province, about 30 plants belonging to 21 families are used by the traditional healers for the treatment of TB and associated diseases (Lawal et al., 2014).

1.1.5 Need for the discovery of new drugs from herbal origin

The emergence of drug resistant strains of *Mycobacterium tuberculosis* has complicated the treatment of TB. Due to the presence of "cross resistance" no single drug or combined therapy is able to control TB completly. This type of resistance is developed only against purified chemical compounds. Any single purified chemical compound has the ability to produce resistance in pathogens. Mycobacteria possess the capability to digest purified drugs by modifying their receptor structure according to the drugs, thus, slowly adapt and develop resistance against the anti-tubercular agents. However, pathogens do not induce the problem

of drug resistance to herbal medicines (Khadar, 2013; Mehta et al., 2015). Natural products and/or their semi-synthetic derivatives are therefore, important sources of new chemical compounds that might play an important role in the chemotherapy of tuberculosis (Pavan et al., 2009; Nguta et al., 2015b).

1.1.6 Polyherbal medicines

Polyherbal medicines are mixtures of various herbs which contain multiple active constituents and act synergistically against infections (Bhope et al., 2011). They have been used extensively for the treatment of various diseases for many centuries due to their medicinal and therapeutic application (Aslam et al., 2016). Several studies on the use of polyherbal medicines have revealed that these therapies possess pharmacological functions. For instance, Rajanyamalakadi, a polyherbal preparation which contains three herbal ingredients has been proven to show significant antidiabetic, hypolipidemic and antioxidant properties (Faizal et al., 2009). Also, Polyherbal Health Tonic Tea used for the treatment of an array of diseases affecting humans and Sanjivani Vati used for the treatment of cough and cold have been shown to possess significant pharmacological activities (Adeneye and Benebo, 2009; Gulati et al., 2010). Other Polyherbal remedies such as Livina, Rhumapar tablet, Diakyur and Sugar Remedy have been proven to contain pharmacological activities (Gairola et al., 2011; Patil et al., 2013; Singhal et al., 2014). These herbal remedies act on multiple targets at the same time to express high effectiveness in a vast number of diseases (Parasuraman et al., 2014).

1.2 Rationale and justification for this study

The utility of the current drug regimens combination for tuberculosis have been limited due to incompliance issues of patients, which has resulted into the rise of strains that are resistant to some or the entire first and second-line antibiotics (Hundeiker, 2010). Due to the resistance of *Mycobacterium tuberculosis* to commonly prescribed antimicrobials, relatively high cost and limited access to synthetically derived drugs, communities most especially in Africa have relied on the use of herbal medicine for the treatment of their ailment (WHO, 2003; Orodho et al., 2011). In the continuing search for effective treatment, polyherbal remedies are used as alternative medicines in the Eastern Cape Province of South Africa. It is important to document the ingredients used in the formulations, as the information would be very important in the development of serious armament for the treatment of tuberculosis.

Also, with the ever increasing use of polyherbal medicines and their global market expansion, safety has become a major concern for both health authorities and the public in many countries (WHO, 2007). Several investigations have shown that these remedies are associated with a broad variety of residues and contaminants such as microbial agents and heavy metals (Candlish et al., 2001; Govender, et al., 2006; Alwakeel, 2008; Abba et al., 2009; Kaume et al., 2012; Ting et al., 2013; Noor et al., 2014). Evaluation of the microbial contamination of these remedies is neccessary to justify their folkloric usage. There is equally a need to conduct toxicological evaluation on these herbal formulations in order to acquire their maximum benefits and safety, even though they might have been proven to be efficacious in pharmacological studies or by clinical evaluation (Tatke et al., 2012).

With the increasing incidence of drug-resistant strains of tuberculosis each year in South Africa (Hughes and Osman, 2014), coupled with the suppression of human defence mechanism during the course of active tuberculosis, many patients become vulnerable to pathogenic and opportunistic pathogens; and, as such, acquires fungal infection in addition to

bacterial, viral and parasitic infections (Sunita and Mahendra, 2008; Querido et al., 2011). Scientific validation of the herbal medicines against *M. tuberculosis* and other associated pathogen is thus necessary in proving their effectiveness. Also, in order to authenticate the polyherbal remedies used for the treatment of tuberculosis in the Eastern Cape Province, evaluation of the microbial containants, toxicity, antimicrobial and mineral analyses were carried out to validate their use and guarantee the safety of the users. This reasearch will not only promote the use of traditional medicines in South Africa but also in other countries.

1.3 Objectives of the study

The overall objective of this study was to document the polyherbal medicines that are used for the treatment of tuberculosis, identify the bacteria and fungi present, determine the toxicity, nutritive properties and antimicrobial activity of the polyherbal medicines in the Eastern Cape Province, South Africa.

The specific objectives are:

1. To carry out an ethno-medicinal survey of polyherbal medicines used for the treatment of tuberculosis in the Eastern Cape, South Africa. Such documentation includes the names of the plants including the non-herbal inclusions, part used, method of preparation and the dosage of polyherbal formulations used for the treatment of tuberculosis.
2. To identify the different bacterial contaminants present in the herbal medicines using rapid 16S rRNA technique.
3. To identify the different fungal populations in the polyherbal medicines.
4. To determine the toxicity potential of the herbal remedies using brine shrimp test.
5. To determine the anti-*Mycobacterium tuberculosis* activity of the polyherbal medicines.

6. To determine the effect of the remedies on opportunistic bacterial and fungal pathogens of humans infected with tuberculosis.
7. To evaluate some important vitamins and mineral nutrients present in the polyherbal medicines.

1.4 Description of the study area

This study was carried out in five communities within the Amathole District Municipality of the Eastern Cape Province, South Africa (Figure 1.2). The area falls within latitudes 30°00′ to 34°15′S and longitudes 22°45′ to 30°15′E. It is bounded by the sea on the East and the drier Karroo (semi-desert vegetation) in the West. The elevation ranges from sea level to approximately 2,200 m in the North of the province. The Amathole District Municipality lies at the heart of the Eastern Cape Province. The District stretches from the Indian Ocean coastline in the South to the Amathole Mountains in the North, and from Mbolompo Point (south of the Hole-in-the-Wall along the Transkei Wild Coast) in the East to the Great Fish River in the West. Presently, about 1.7 million people living in the study area (Afolayan 2003) is made up of Africans (91%), coloureds (3%) and whites (6%). The main tribes of the area are Xhosa-speaking people who are divided into several tribes with related but distinct heritages (Dyubeni and Buwa, 2012).

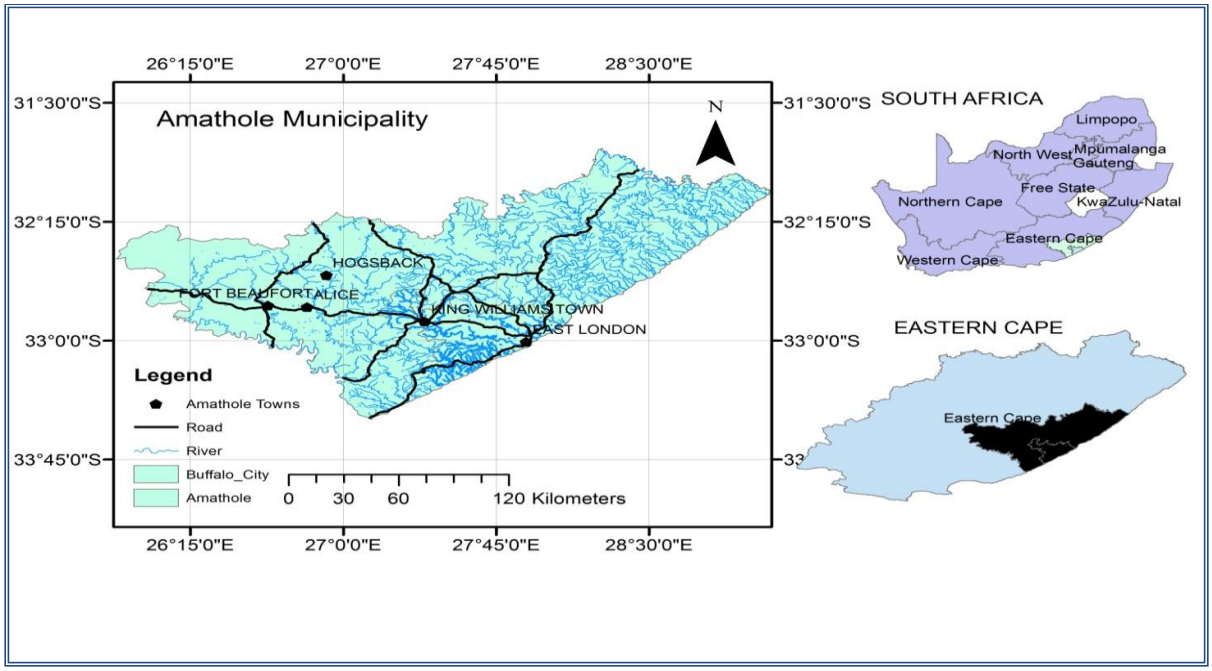

Figure 1.3: Map of Amathole District Municipality (Famewo et al., 2016)

1.5 The structure of the thesis

This thesis is composed of discrete chapters that have been published, accepted or under review in various peer-reviewed accredited journals. The introduction and literature review are presented in Chapter 1. The ethno-medicinal survey of polyherbal medicines used for the treatment of tuberculosis in Amatole District Municipalities of the Eastern Cape Province of South Africa is presented in Chapter 2. The different bacterial contaminants identified in the remedies are reported in Chapter 3 while Chapter 4 is composed of the report of the fungal contaminants present in the polyherbal medicines. The anti-*Mycobacterium tuberculosis* activity of the nine remedies is reported in Chapter 5 while Chapter 6 deals with the effect of the remedies on opportunistic bacterial and fungal pathogens of humans infected with tuberculosis. In Chapter 7, the toxicity potential of the herbal remedies using brine shrimp test was reported. Chapter 8 accounts for the vitamins and mineral nutrients composition present in the polyherbal medicines. The general discussion, conclusions and recommendations emanating from this study are presented in Chapter 9.

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CHAPTER TWO

Ethno-medicinal documentation of polyherbal medicines used for the treatment of tuberculosis in Amathole District Municipality of the Eastern Cape Province, South Africa

CHAPTER TWO

TABLE OF CONTENT

Contents **Page No**

PHARMACEUTICAL BIOLOGY, 2017
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RESEARCH ARTICLE

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Ethno-medicinal documentation of polyherbal medicines used for the treatment of tuberculosis in Amathole District Municipality of the Eastern Cape Province, South Africa

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ABSTRACT

Context: Tuberculosis (TB) has remained a devastating global public health problem. In the continuing search for effective treatment, polyherbal remedies used as alternative medicines in the Eastern Cape Province of South Africa were surveyed.

Objective: The survey collected information and documents the list of ingredients such as the name of the plants used including the non-herbal inclusions, type and dosage of polyherbal formulations used for the treatment of TB.

Materials and methods: The survey was conducted over a period of 6 months using semi-structured questionnaires amidst informal conversations with the traditional healers in five communities in the study area. The chosen study area is the third infected Province with TB in South Africa.

Results: A total of nine polyherbal preparations were collected. Information on the parts of the plant used, mode of preparation and the dosage used were documented. In total, the herbs belong to 20 families of which Apiaceae, Liliaceae, Strychnaceae, Rutaceae and Hypoxidaceae are the most prominent. However, members of Apiaceae were commonly mentioned for the preparation of the remedies. The two most frequently used plants were *Allium sativum* L. (Liliaceae) and *Strychnos decussata* (Pappe) Gilg. (Strychnaceae). Rhizome was the commonest parts used, followed by the roots and barks.

Conclusions: This paper provides significant ethno-medicinal information on polyherbal medicines used for the treatment of TB in the study area. The therapeutic claims made on medicinal plants used for the preparations are well supported by the literature, with many of the species having antimicrobial properties.

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Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* has remained one of the most prevalent causes of mortality in developing nations, especially in the Asian and African continents. The disease remains a big problem in these countries probably due to inadequate means for the management and treatment of the disease. According to World Health Organization (2002), about 95% of the approximately eight million cases of TB occur each year in Africa having the highest incidence. However, South Africa is the third highest incidence country after India and China with about 80% of the population infected with the disease. This rate has increased by 400% over the past 15 years most especially among the people living with human immunodeficiency virus (HIV) (Lall & Meyer 1999; Green et al. 2010).

The conventional medical treatment for TB consists of a regimen of antibiotics taken over the course of several months. For this reason, many people especially those living in the rural communities do not strictly adhere to the plan for effective treatment; thus making the disease difficult to treat with conventional medicines (Lange et al. 2014). In addition, the side effects such as gastrointestinal upset, hepatitis, drug interactions and hearing loss associated with the consumption of these drugs have discouraged many people from continual use of the orthodox medicines (Laxminarayan et al. 2006). Also, the emergence of multi-drug resistant tuberculosis (MDRTB: resistant to the two most effective first-line drugs, isoniazid, and rifampin), extensively drug-resistant tuberculosis (XDRTB: MDRTB with additional resistance to at least one fluoroquinolone and one injectable drug) and totally drug-resistant (TDR) tuberculosis have made TB exacerbated, thus becoming a global health problem. The continual resistance of *M. tuberculosis* to commonly prescribed antimicrobials has made people fall back on herbal medicines for various therapeutic purposes (Orodho et al. 2011).

The inhabitants of the Eastern Cape Province have a long history of traditional plant usage for the treatment of various diseases including TB (Grierson & Afolayan 1999). In fact, about 30 plants belonging to 21 families are used by the traditional healers for the treatment of TB and associated diseases (Lawal et al. 2014). These plants are commonly combined with polyherbal remedies, which are prescribed in different formulations. However, the lists of ingredients used for the polyherbal formulations in the study area are yet to be documented. This information is very important in the development of serious armament for the treatment of TB, since a greater number of TB patients depend on traditional herbalists for their medical needs. Therefore, the aim of this study is to collect information and

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document the list, type, dosage and nature of the polyherbal medicines used for the treatment of TB in this Province, and to collect the information on the ingredients of the polyherbal formulations such as the name of the plants used including the non-herbal inclusions present in each remedy.

Materials and methods

Study area

The present study was carried out in five communities within the Amathole District Municipality of the Eastern Cape Province, South Africa (Figure 2.1). The area falls within latitudes 30°00′ to 34°15′S and longitudes 22°45′ to 30°15′E. It is bounded by the sea on the east and the drier Karroo (semi-desert vegetation) in the west. The elevation ranges from sea level to approximately 2200 m in the north of the province. The Amathole District Municipality lies at the heart of the Eastern Cape Province. The District stretches from the Indian Ocean coastline in the south to the Amathole Mountains in the north, and from Mbolompo Point (south of the Hole-in-the-Wall along the Transkei Wild Coast) in the east to the Great Fish River in the west. Presently, about 1.7 million people live in the study area (Afolayan 2003) including Africans (91%), coloreds (3%) and whites (6%). The main tribes of the area are Xhosa-speaking people(s) who are divided into several tribes with related but distinct heritages (Dyubeni & Buwa 2012).

Data collection

The survey on the polyherbal medicines was conducted between March and August 2015 through semi-structured questionnaires amidst informal conversations with the traditional healers who use medicinal plants for the treatment of TB (Ajibesin et al. 2012; Asiimwe et al. 2013). The survey was carried out among the herb healers because they inherited the knowledge from their forefathers, therefore, passing it from one generation to another generation. The interviews were conducted in Xhosa, the local language of the informants, and English. The remedies were already prepared with water by the herbal healers into clean 2 L containers. They were purchased and the following information was collected for proper documentation; the local name of the herbs used for the polyherbal formulations, plant parts used, methods of preparation, modes of administration of the herbal remedy, doses and duration of treatment.

Results and discussion

This study revealed that polyherbal remedies play an important role in healthcare delivery in South Africa, especially among the people living in the rural settings of the Eastern Cape Province. The application of these remedies in the management or treatment of diseases covers a wide range of conditions from chronic conditions, psychosocial problems, acute conditions, generalized pain, TB and HIV infection and acquired immune deficiency syndrome (AIDS). Ethnobotanical surveys have extensively studied South African populations of adults and children with several illnesses (Bodkin 2003).

A total of nine polyherbal medicines used for the treatment of TB were recorded and collected from the study area (Table 2.1); these are from East London (EL), King Williams Town (KWT) and Fort Beaufort (FB). Others are from Alice (AL) and Hogsback (HB). Each remedy was labelled and coded according to the place of collection; namely King Williams Town site A (KWTa), King Williams Town site B (KWTb), King Williams Town site C (KWTc), Hogsback first site (HBfs), Hogsback second site (HBss), Hogsback third site (HBts), East London (EL), Alice (AL) and Fort Beaufort (FB). The small number of remedies obtained in this study was because only a few traditional healers treat TB. They claim to have acquired the knowledge from their ancestors, and this knowledge has been transferred from one generation to another. The herbal healers in the Province diagnosed TB based on patients' signs and

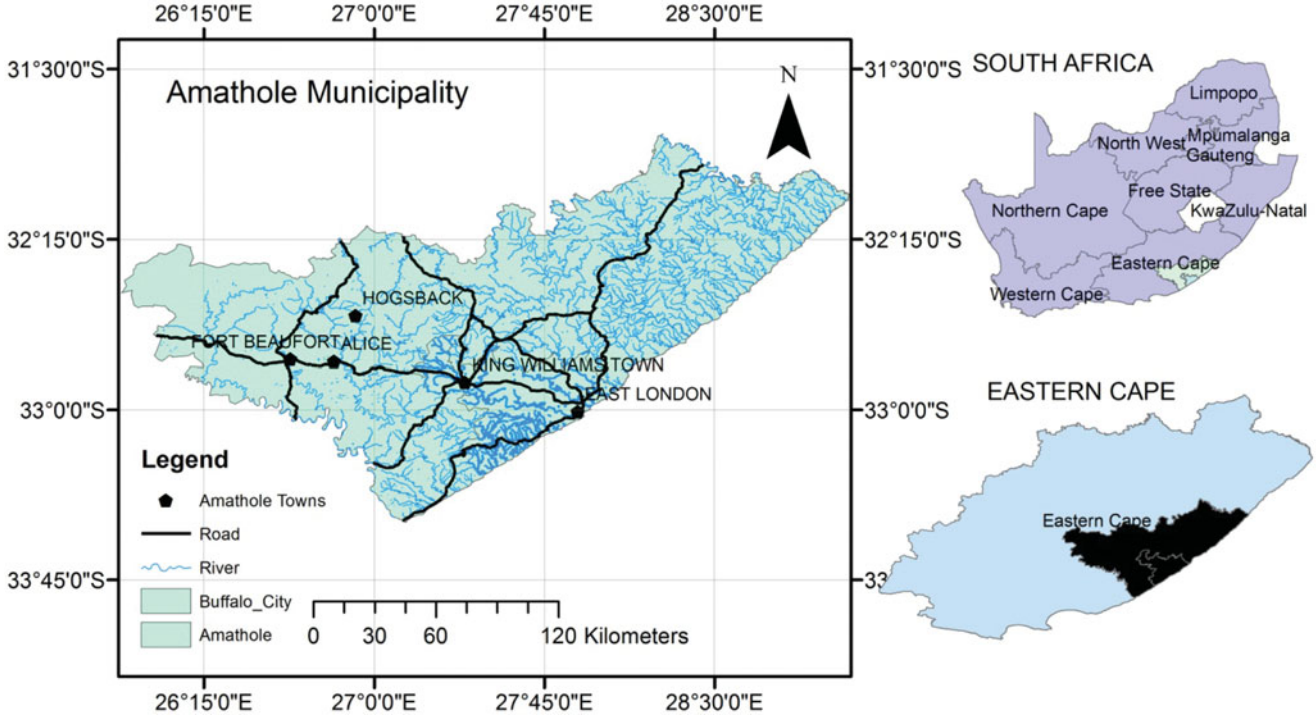

Figure 2.1. Map of Amathole District Municipality. (Source: Famewo et al. 2016)

symptoms before commencing with the treatment. Patients are carefully observed and symptoms such as blood in the sputum, prolonged coughing, chest pain and breath shortness including weight loss are taken as confirmation of TB.

The polyherbal remedies in the study area were prepared from 24 plants. The six most commonly mentioned plant families used in the preparation of the polyherbal formulations in terms of number and percentage are Apiaceae [5(25%)], followed by Liliaceae [4(20%)], Strychnaceae [4(20%)], Rutaceae [4(20%)], Solanaceae [2(10%)] and Primulaceae [2(10%)] (Figure 2.2). Members of Apiaceae have been previously cited as the most commonly used in this Province not only for the treatment of TB but also as an antidote for influenza, hypertension and to expel intestinal worms (Bisi-Johnson et al. 2010). The therapeutic claims made on most of the medicinal plants used for the preparation of these remedies are well supported by the literature, with many of the species having antimicrobial properties (Buwa & Afolayan 2009; Green et al. 2010).

The most frequently used plant parts in this study was rhizomes [10(27%)], followed by bark [8(22%)], and roots [8(22%)]. Others are leaves [6(16%)], corms (3(8%)] and vegetable [2(5%)] as shown in Table 2.1. The four most frequently used plants are *Allium sativum* L. (Liliaceae) [4(17%)], *Strychnos decussata* (Pappe) Gilg. (Strychnaceae) [4(17%)], *Daucus carota* L. (Apiaceae) [3(13%)] and *Hypoxis argentea* (Fiscand) (Hypoxidaceae) [3(13%)]. Other plants such as *Agathosma betulina* (Berg) (Rutaceae) [2(8%)], *Capsicum annuum* L. (Solanaceae) [2(8%)] and *Rapanea melanophloeos* L. (Primulaceae) [2(8%)] are used for a few polyherbal preparations in the Province as represented in Figure 2.3.

Table 2.1. Polyherbal medicines used for the treatment of TB in Amathole District Municipality, Eastern Cape Province, South Africa.

Name code	Ingredients	Botanical name	Family	Parts used	Methods of preparation, administration and dosage
AL	Mountain garlic	*A. sativum* (L.)	Liliaceae	Rhizome	Infusion; Take 100 mL of the herbal mixture orally twice in a day for a period of 5–8 weeks.
	Mlomo mnandi	*Glycyrrhiza glabra* (L.)	**Fabaceae**	Root	
	Red carrot	*Daucus carota* (L.)	Apiaceae	Root	
	Inongwe	*Hypoxis argentea* (Fiscand)	Hypoxidaceae	corms	
	Mnonono	*S. decussate* (Pappe) Gilg	Strychnaceae	Bark	
	River pumpkin	*Gunnera perpensa* (L.)	Gunneraceae	Rhizome	
	Herbal menthol leaf	*Mentha piperita* (L.)	Lamiaceae	Leaf	
	Herbal buchu water	*Agathosma betulina* (Berg)	Rutaceae	Leaf	
EL	Inongwe	*Hypoxis argentea* (Fiscand)	Hypoxidaceae	corms	Infusion; Take 100 mL of the remedy orally thrice in a day for a period of 5–8 weeks.
	Intelezi	*Haworthia reinwardtii* (Haw)	Xanthorrhoeaceae	Leaf	
	Ngcambumvuthuza	*Ranunculus multifidus* (Forssk)	Ranunculaceae	Root	
	Inqwebeba	*Albuca flaccid* (Jacq.)	Asparagaceae	Leaf	
	Iqwili	*Alepidea amatymbica* (Eckl. & Zeyh.)	Apiaceae	Rhizome	
FB	Buchu leaf	*Agathosma betulina* (Berg)	Rutaceae	Leaf	Decoction; Take 75 mL of the remedy orally thrice in a day for a period of 5–8 weeks.
	Mountain garlic	*A. sativum* (L.)	Liliaceae	Rhizome	
	Ginger	*Zingiber officinalis* (L.)	**Zingerberaceae**	Rhizome	
	Chilli pepper	*Capsicum annuum* (L.)	Solanaceae	Vegetable	
KWTa	Maphipha	*Rapanea melanophloeos* (L.)	Primulaceae	Bark	Infusion; Take half a cup thrice in a day for a period of 5–8 weeks.
	Mnonono	*S. decussate* (Pappe) Gilg	Strychnaceae	Bark	
	Ixonya	*Kniphofia drepanophylla* (Baker)	Asphodelaceae	Root	
	Inongwe	*Hypoxis argentea* (Fiscand)	Hypoxidaceae	Corms	
	Sicimamlilo	*Pentanisia prunelloides* (Klotzsch)	Rubiaceae	Rhizome	
	Iphuzi	*Centella eriantha* (Rich.)	Apiaceae	Rhizome	
KWTb	Umdlavuza	*Lauridiatetragonia* (L.f.)	Celastaceae	Root	Infusion; Take 100 mL of the polyherbal remedy orally thrice in a day for a period of 5–8 weeks
	Mnonono	*S. decussate* (Pappe) Gilg	Strychnaceae	Bark	
	Inceba emhlophe	*Hermannia* sp. (L.)	Malvaceae	Root	
KWTc	Mnonono	*S. decussate* (Pappe) Gilg	Strychnaceae	Bark	A little quantity of the herb must be chewed immediately after the polyherbal remedy in KWT B has been administered, thrice in a day for a period of 5–8 weeks.
HBfs	Red carrot	*Daucus carota* (L.)	Apiaceae	Root	Infusion; Take 75 mL of the polyherbal remedy orally thrice in a day for a period of 3–5 weeks
	Mlungu mabele	*Zanthoxylum capense* (Thunb.)	Rutaceae	Bark	
	Calmoes	*Acorus calamus* (L.)	Acoraceae	Rhizome	
	Mountain garlic	*A. sativum* (L.)	Liliaceae	Rhizome	
HBss	Buchu leaf	*Agathosma betulina* (Berg)	Rutaceae	Leaf	Decoction; Take 75 mL of the herbal mixture orally thrice in a day for a period of 3–5 weeks
	Chilli pepper	*Capsicum annuum* (L.)	Solanaceae	Vegetable	
HBts	Maphipha	*Rapanea melanophloeos* (L.) Mez	Primulaceae	Bark	Infusion; Take 75 mL of the herbal mixture orally thrice in a day for a period of 3–5 weeks
	Red carrot	*Daucus carota* (L.)	Apiaceae	Root crop	
	Uroselina	*Cinnamomum camphora* (L.) J. Presl	Lauraceae	Bark	
	Mountain garlic	*A. sativum* (L.)	Liliaceae	Rhizome	

AL: Alice; EL: East London; FB: Fort Beaufort; KWTa: King Williams Town site A; KWTb: King Williams Town site B; KWTc: King Williams Town site C; HBfs: Hogsback first site; HBss: Hogsback second site; HBts: Hogsback third site.

The importance of Fabaceae lies in their effectiveness in the treatment of a wide variety of human ailments such as allergy, cough, hiccups, stomach ulcers, viral fevers, wounds and swellings (Padal et al. 2013). They also possess high level of biological activity due to the variety of chemically active constituents such as tannins, flavonoids, alkaloids, and terpenes present in members of this family (Molares & Ladio 2011).

Zingiberaceae is rich in substances having therapeutic value such as terpenoids, tannins and flavonoids. The rhizomes of this family are aromatic, tonic and stimulant. They are used as antimicrobial, antiarthritic, antioxidant, anticancer, antiinflammatory, antidiabetic, neuroprotective and larvicidal agents (Victório 2011).

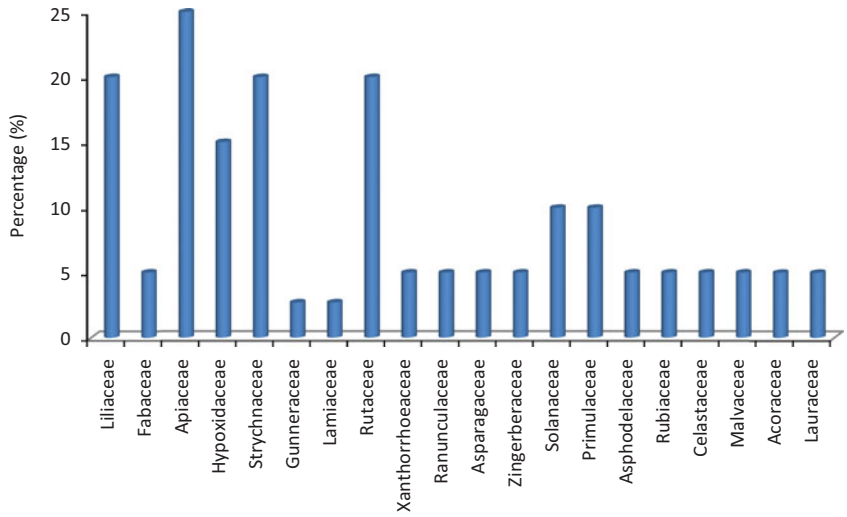

Figure 2.2. Frequency of the most used plant families in the preparation of polyherbal medicines for the treatment of TB in the study area.

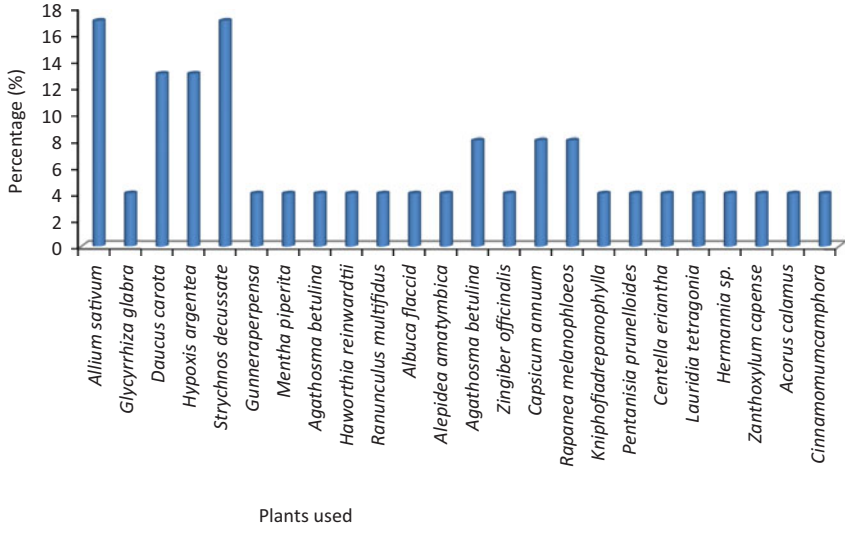

Figure 2.3. Occurrence of plant species used for the preparation of polyherbal medicines for the treatment of TB in the study area.

The polyherbal medicines were prepared mainly by infusion [6(67%)] and decoction [2(22%)] with the exception of *S. decussate,* which should be chewed immediately after KWTb remedy has been administered (Table 2.1). This is an indication that the Eastern Cape traditional herbal healers use water mainly for the preparation of anti-TB treatments. These methods of extraction have been adopted from the ancient time. They seem to yield active principles required to treat TB (Nguta et al. 2015). The internal method of administration, which is by oral, was the main method of administrating all the remedies.

The diverse uses of each plant can be explicated by the fact that, a single plant can serve many medicinal purposes or perform different functions (Lawal et al. 2010). However, the combination of these herbs in polyherbal medicines probably results in better therapeutic activities and reduces the toxicity of such remedies. Naturally, polyherbal remedies contain multiple active constituents, which act synergistically against infections (Bhope et al. 2011). A similar study by Amodu et al. (2013) revealed that polyherbal medicines were effective against *Mycobacterium tuberculosis*. Probably, these therapies have gained popularity in both developed and developing countries because of their natural origin and fewer side effects for the treatment of various chronic and acute ailments (Ahmad et al. 2006; Benzie & Wachtel-Galor 2011). Polyherbal medicines are current pharmacological principle having the advantage of producing maximum therapeutic efficacy with minimum side effects to the consumers (Ebong et al. 2008). The cross-cultural acceptance and use of polyherbal remedies in different geographical zones is an indication of the potential of polyherbal as future sources of new classes of drugs against TB.

Conclusions

This paper provides significant ethno-medicinal information on polyherbal medicines used for the treatment of TB in Amathole District Municipality of Eastern Cape Province. Africa is endowed with a biodiversity of medicinal plants, many of which are currently used in the traditional management of TB. The study shows that people in the study area still depend on polyherbal medicines for the treatment and management of TB. The documented remedies reflect rich ethno-medicinal knowledge in the province.

However, further test are required to validate the ethno-medicinal usage of these polyherbal remedies as anti-TB agents.

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Disclosure statement

The authors report that they have no conflicts of interest.

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CHAPTER THREE

Identification of bacterial contaminants in polyherbal medicines used for the treatment of tuberculosis in Amatole District of the Eastern Cape Province, South Africa using Rapid 16S rRNA technique

CHAPTER THREE

TABLE OF CONTENT

Chapters **Page No**

Famewo et al. Journal of Health, Population and Nutrition (2016) 35:27
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RESEARCH ARTICLE Open Access

Identification of bacterial contaminants in polyherbal medicines used for the treatment of tuberculosis in Amatole District of the Eastern Cape Province, South Africa, using rapid 16S rRNA technique

Elizabeth Bosede Famewo, Anna Maria Clarke and Anthony Jide Afolayan*

Abstract

Background: Polyherbal medicines are used for the treatment of many diseases in many African and Asian communities. With the increasing use of these remedies, several investigations have shown that they are associated with a broad variety of residues and contaminants. This study investigates the presence of bacteria in the polyherbal medicines used for the treatment of tuberculosis (TB) in the Eastern Cape Province of South Africa.

Methods: Bacterial DNA was extracted from the polyherbal medicines, and a fragment of the bacterial 16S rRNA gene was amplified by PCR with universal primers 27F and 518R. The amplicons were visualised on agarose gel electrophoresis, followed by end repair and adaptor ligation. They were further purified and quantified using Library Preparation kit NEBNext® UltraT DNA Library Prep Kit for Illumina, and the amplicons were run on illumina's MiSeq platform.

Results: Different bacterial species were identified in all each of the polyherbal medicines. Generally, the most prominent and common bacteria recovered from all the samples were *Bacillus* sp., *Enterobacter* sp., *Klebsiella* sp., *Rahnella* sp., *Paenibacillus* sp., *Clostridium* sp. and *Pantoea* sp. Others are *Pseudomonas* sp., *Raoultella ornithinolytica*, *Salmonella enterica* and *Eubacterium moniliforme*.

Conclusions: This study, thus, revealed the presence of pathogenic and non-pathogenic bacteria in the polyherbal medicines used for the treatment of tuberculosis in the study area. The implications of the findings are discussed in relation to the health care of the patients of tuberculosis in the study area, having in mind that they are immunocompromised individuals.

Keywords: Polyherbal medicines, Tuberculosis, Bacteria, Public health, Sequencing

Background

Polyherbal medicines have been used for various therapeutic purposes as far back as the origin of mankind. In South Africa, it is estimated that three million people currently use herbal remedies (polyherbal medicines) for their health care purposes especially in the treatment of diarrhoea, diabetes, stomach illnesses, wound infections and tuberculosis [1–3].

About 1 % of the South Africa population is estimated to develop tuberculosis yearly [4]. The country accounts for one quarter of the global burden of HIV-associated tuberculosis (TB) [5]. This is stimulated by the high rates of latent tuberculosis infection and the increase in the prevailing rates of infection [4]. In fact, about 50 % of people among the age group 15 and 77–89 % of adults have evidence of latent TB infection [6, 7]. The use of the current drug regimen combinations for TB is limited due to patients' non-compliance, which has resulted in the rise of strains that are resistant to some

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or the entire first and second-line antibiotics [8]. The emergence of multi-drug resistant tuberculosis (MDRTB), extensively drug resistant tuberculosis (XDRTB), and to-tally drug resistant tuberculosis (TDR) has exacerbated the global health problem [9, 10]. Due to the high resist-ance of *M. tuberculosis* to commonly prescribed antimi-crobials, relatively high cost and limited access to synthetically derived drugs, most communities especially in Africa still rely on the use of polyherbal medicine for the treatment of their ailments [11, 12]. Yet, these medi-cines have been reported to contain a number of microbial and heavy metal contaminants [13–16].

Medicinal herbs frequently harbour a large number of microbes originating from the soil, and these microorganisms normally adhered to leaves, stems, flowers, seeds and roots of plants [17]. The contamination of herbal products with Enterobacteriaceae, *Bacillus* spp., *Salmonella* spp., *Staphylococcus aureus*, *Penicillium* spp. and *Aspergillus* spp. have been reported by [18]. Also, elevated levels of bacterial and fungal contaminants, such as *Escherichia coli*, yeast, *Penicillium* spp., *Aspergillus* and *Fusarium*, were observed in herbs and spices by [14, 19, 20]. The presence of these contaminants in the herbal products might adversely affect the health status of the consumers due to their immunocompromised conditions. Microbial infections have posed a health problem throughout the world, thus, the safety of the consumers of herbal products is of utmost importance.

To the best of our knowledge, the microorganisms present in some of the polyherbal medicines used for the treatment of TB in Amathole District Municipality of the Eastern Cape Province have not been investigated despite the mass consumption of the medicines. Most of these polyherbal remedies are prepared in the form of concoctions or infusions and are left at room temperature over a long period of time depending on how rapid the patients respond to treatment. During this period, the mineral elements present in these remedies may facilitate the growth of microorganisms. Thus, this study therefore aimed at identifying different bacteria present in some of the polyherbal medicines used for treatment of TB in the study area using molecular based technique.

Methods

Sample collection

A total of nine polyherbal medicines used for the treatment of TB were purchased from the traditional herbal sellers in five communities, namely East London (EL), King Williams Town (KWT), Hogsback (HB), Alice (AL) and Fort Beaufort (FB) as shown in Fig. 3.1. Each remedy was labelled and coded according to the place of collection, viz: King Williams Town site A (KWTa), King Williams Town site B (KWTb), King Williams Town site C (KWTc), Hogsback first site (HBfs), Hogsback second site (HBss), Hogsback third site (HBts), East London (EL), Alice (AL) and Fort Beaufort (FB). The small number of remedies obtained in this study was due to the fact that only a few traditional healers treat and sell the remedies for TB. They claim to have acquired the knowledge from their ancestors, and this knowledge is been transferred from one generation to another. The samples were then transported to Medicinal Plants and Economic Development (MPED) Research Centre for analysis.

DNA extraction

The total bacterial DNA was extracted in a clean and sterilized environment using ZR Fungal/Bacterial MiniPrep™ Kit (Zymo Research, USA). The method of [21] with slight modification was used for the extraction. One millilitre of each sample was pipetted into sterile eppendorff tubes and centrifuged at 12,500 rpm for 10 min. The supernatant was discarded, and the cell pellets were collected. The protocol in the extraction kit was followed.

PCR amplification of bacterial DNA

Polymerase chain reaction (PCR) was performed using the extracted DNA from each of the samples. The bacterial 16S rRNAs were amplified using the oligonucleotide primers 27F (5′-GGT AGA GTT TGA TCC TGG CTC AG-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′). The 16S rRNA gene contains nine variable regions (designated V1 to V9) of which we chose the V1-V3 regions, which has previously proven useful in research-oriented metagenomic surveys [22]. A total reaction volume of 25 μL was used, which contained 12.5 μL Master Mix (Thermo Scientific, EU Lithuania), 1 μL each of 10 μM of both forward and reverse primer solutions (Inqaba Biotech, SA), 5.5 μL of nuclease free water and 5 μL template DNA. Reactions was performed in the thermocycler (Bio-Rad Mycycler, USA) using the following cycling conditions: initial denaturation at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 1.5 min and final extension at 72 °C for 10 min [23]. In order to confirm the products size, 5 μL of the amplicons was analysed by gel electrophoresis in 1 % agarose (Merck, SA) stained with 3 μL ethidium bromide (Sigma-Aldrich, USA). A 100 bp DNA ladder for 16S rRNA (Thermo Scientific, (EU) Lithuania) was included for band size estimation purposes. All gels were run in 0.5X TBE buffer at 95 V for 1 h and visualised by UV trans-illumination (Alliance 4.7, France).

Purification of 16S rRNA gene amplicons and sequencing

The bacterial 16S rRNA gene amplicons were purified with the Zymoclean™ Gel DNA Recovery kit (ZymoResearch

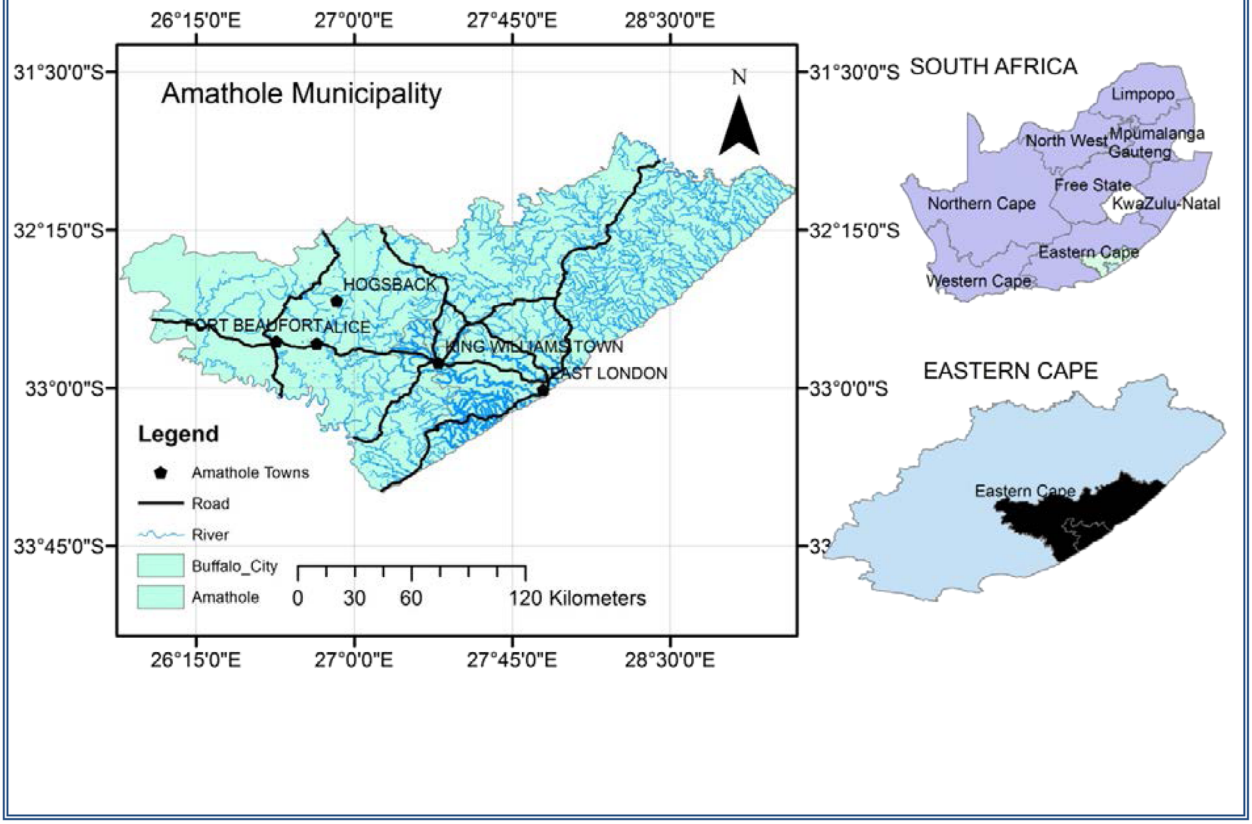

Fig. 3.1 Map of Amathole District Municipality

Corporation, Irvine, USA) and quantified using NanoDrop Fluorospectrometer ND3300 fragment size (Agilent Bioanalyzer 2100) prior sequencing. Followed by end repair and adaptor ligation and quantification of each library using Library Preparation kit (NEBNext® UltraT DNA Library Prep Kit for Illumina), before running them on illumina's MiSeq platform following the amplicon sequencing protocol [24].

Results

Bacterial families and species identified in the remedies

The findings of this study revealed the presence of both pathogenic and non-pathogenic bacteria in the polyherbal medicines used for the treatment of tuberculosis in the Eastern Cape Province of South Africa. All the identified bacteria in the therapies belong to 12 families. Only one of the families could not be identified (Fig. 3.2). However, the prevalent families in all the polyherbal medicines are the Enterobacteriaceae, Bacillaceae and Paenibacillaceae (Fig. 3.2).

While the majority of the bacteria identified in KWTa remedy are Enterobacteriaceae (68 %), others were Bacillaceae (23 %), with 9 % remaining unknown (Fig. 3.3a). In the same vein, Enterobacteriaceae are the dominant organisms in KWTb remedy and only 8 % remaining unknown (Fig. 3.3b). The family Bacillaceae dominate KWTc therapy (66 %), whereas only 3 % of the bacteria are unknown (Fig. 3.3c). The identities of the majority of bacteria present in the herbal medicines sourced from AL and EL were unknown (84 and 86 %, respectively), with the remaining bacteria belonging to 16 and 5 families in AL and EL samples, respectively (Figs. 3.3d, e). A high number of bacteria identified in FB belong to Paenibacillaceae (98 %) as against a few other remedies where their population is insignificant (Fig. 3.3f). Similarly, HBfs, HBss and HBts remedies were dominated by Enterobacteriaceae (Figs. 3.3g–i).

This study further investigated the bacterial species present in each of the polyherbal medicines. The overall blast output revealed that these polyherbal remedies are contaminated with different bacterial genera and species including *Bacillus* sp., *Klebsiella* sp., *Rahnella* sp., *Paenibacillus* sp., *Enterobacter* sp., *Pantoea* sp., *Clostridium* sp., and a few unknown (Table 3.1). Some of these bacteria that are clinically pathogenic to human include *Raoultella ornithinolytica*, *Rahnella aquatilis*,

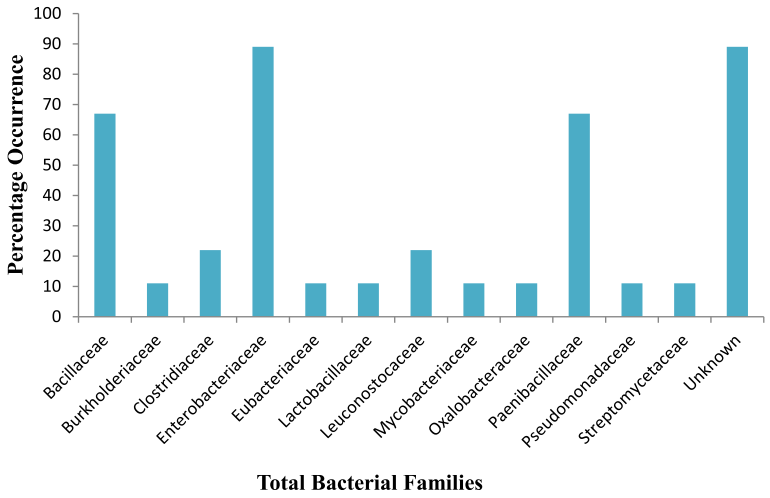

Fig. 3.2 Percentage occurrence of each bacteria family identified in all the polyherbal remedy

Bacillus anthracis, *Bacillus cereus*, *Salmonella enteric*, *Enterobacter cloacae*, *Klebsiella oxytoca* and *Klebsiella pneumonia*. Others such as *Enterobacter asburiae*, *Paenibacillus polymyxa*, *Pantoea rwandensis*, *Klebsiella variicola* and *Pseudomonas* sp. are opportunistic pathogens causing opportunistic infections in individuals with impaired immunity.

Discussion

The use of polyherbal medicines for the treatment of various diseases is still a significant practice in the developing countries including South Africa. With the popularity and global market expansion, the safety of herbal products has become a major concern to public health [25]. In this study, all the polyherbal therapies used for the treatment of tuberculosis are orally consumed in the form of water-extracted remedies. Naturally, the ingredients used for the preparation of these remedies are not usually sterilized before soaking in water, hence the presence of different bacteria species and families identified in the polyherbal medicines. The main source of contamination could be from the soil, water, plant or other raw materials and the containers used. Since these remedies are not prepared in a sterile manner, another possible source of contamination could be contaminants from the personnel(s), unhygienic production conditions, during harvesting, drying and storage. In addition, environmental factors such as temperature, humidity and extent of rainfall during pre-harvesting and post-harvesting periods can influence the microbial contamination of these medicinal herbal [26].

The presence of Bacillaceae, such as *B. cereus* and *B. anthracis*, and Enterobacteriaceae including *R. ornithinolytica*, *Rahnella* sp., *Klebsiella* sp. and *Enterobacter* sp. in these polyherbal medicines is a cause for concern. Some of these bacteria are pathogenic to humans while others are opportunistic pathogens (Table 3.1) but could be serious public health hazard causing opportunistic infection and reduces the immunity of the immune-suppressed consumers. *R. ornithinolytica* (formerly named *Klebsiella ornithinolytica*) was identified in KWTa remedy. This bacterium is Gram-negative, non-motile, encapsulated and aerobic bacillus [27]. Though an uncommon human pathogen, about 86 infectious cases of this organism have been reported [28]. This pathogen has been linked to bacteremia [29, 30], sepsis [31], acute suppuration of the pancreatic duct [32], soft tissue infection [33], enteric fever [34], renal cysts [35] and urinary tract infection [36]. Also, it expresses chromosomal class A β-lactamases, which confer resistance to ampicillin and other aminopenicillins [28].

R. aquatilis was identified in AL remedy, KWTa, KWTb and KWTc remedies (Table 3.1). The bacterium is a faculta-tively anaerobic, nitrogen-fixing and Gram-negative rod-shaped organism. It has been reported to cause infections such as bacteremia, sepsis, respiratory infection, urinary tract infection and wound infections most especially in immune-suppressed individuals [37, 38]. Also, about 18 cases of human infection caused by *R. aquatilis* has been reported, and majority of these infections were accompan-ied by diabetes mellitus, alcoholism, cancer and AIDS [38]. The presence of this bacterium in the polyherbal remedies is a cause for concern considering the immuno-compromised status of the patients.

Bacillus species such as *Bacillus cereus*, *B. anthracis* and *B. subtilis* were also identified in the polyherbal remedies. *Bacillus cereus* is a Gram-positive, aerobic-to-facultative, spore-forming rod bacterium bearing close phenotypic and genetic relationships to several *Bacillus* species most especially *B. anthracis* [39]. This bacterium

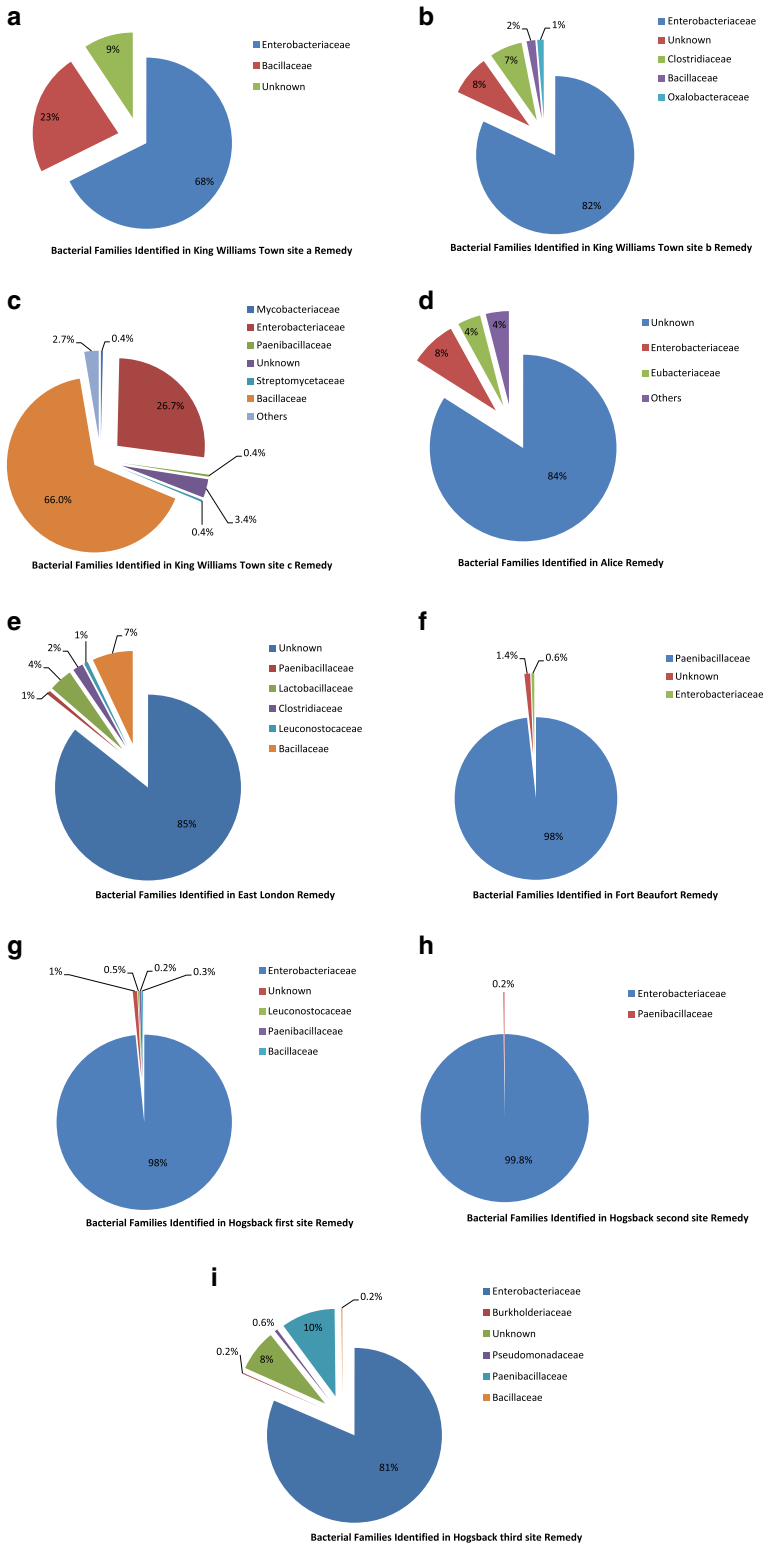

Fig. 3.3 Relative frequencies of contaminating organisms in each polyherbal remedy

Table 3.1 Pathogenic and non-pathogenic bacterial species identified metagenomically from the polyherbal medicines used for the treatment of tuberculosis in the Eastern Cape Province of South Africa

KWTa	KWTb	KWTc	AL	EL	FB	HBfs	HBss	HBts
*[a]*Raoultella ornithinolytica*	*[a]*Rahnella aquatilis*	[nb]*Bacillus thuringiensis*	*[a]*Rahnella aquatilis*	*[b]*Bacillus cereus*	[h]*Paenibacillus* sp.	[a]*Enterobacter* sp.	*[a]*Klebsiella oxytoca*	[nb]*Bacillus subtilis*
*[b]*Bacillus anthracis*	[na]*Klebsiella variicola*	*[a]*Rahnella aquatilis*	[nj]*Eubacterium moniliforme*	[nb]*Lysinibacillus sphaericus*	[nh]*Paenibacillus terrae*	[h]*Paenibacillus* sp.	[a]*Enterobacter* sp.	[nh]*Paenibacillus polymyxa*
*[b]*Bacillus cereus*	*[a]*Klebsiella oxytoca*	[b]*Bacillus* sp.	Uncultured firmicutes	[nk]*Lactobacillus* sp.	[a]*Enterobacter* sp.	[na]*Pectobacterium carotovorum*	[a]Uncultured enterobacter	[na]*Pantoea rwandensis*
*[a]*Klebsiella pneumoniae*	*[b]*Bacillus cereus*	*[b]*Bacillus cereus*	Uncultured bacterium	[ne]*Clostridium thiosulfatireducens*	Uncultured bacterium	[b]*Bacillus* sp.	[na]*Enterobacter cloacae*	[na]*Pantoea agglomerans*
*[a]*Rahnella aquatilis*	*[a]*Klebsiella pneumoniae*	Uncultured bacterium	–	[ne]*Clostridium xylanolyticum*	Uncultured actinobacterium	[nl]*Weissella soli*	[nh]*Paenibacillus polymyxa*	*[a]*Klebsiella pneumoniae*
[a]*Enterobacter* sp.	*[a]*Salmonella enterica*	[na]*Kluyvera intermedia*	–	[h]*Paenibacillus* sp.	–	Uncultured firmicutes	[na]*Citrobacter freundii*	[nm]*Burkholderia xenovorans*
[na]*Klebsiella variicola*	[nf]*Herbaspirillum frisingense*	[ng]*Mycobacterium chelonae*	–	[e]Uncultured clostridium	–	Uncultured bacterium	–	[np]*Pseudomonas* sp.
[na]*Leclercia* sp.	[na]*Enterobacter cloacae*	[nh]*Paenibacillus* sp.	–	[nl]*Weissella soli*	–	–	–	[na]*Pantoea vagans*
[b]Uncultured bacillus	[na]*Enterobacter asburiae*	[ni]*Streptomyces leeuwenhoekii*	–	Uncultured bacterium	–	–	–	[a]*Klebsiella* sp.
[c]*Bacterium nxked5*	[e]Uncultured clostridium	–	–	[nl]*Leuconostoc mesenteroides*	–	–	–	[a]*Pantoea* sp.
[nb]*Bacillus thuringiensis*	[e]*Clostridium* sp.	–	–	[b]Uncultured bacilli	–	–	–	Uncultured bacterium
Uncultured bacterium	[c]*Bacterium mj07*	–	–	–	–	–	–	–
–	[na]*Kosakonia radicincitans*	–	–	–	–	–	–	–
–	[c]*Bacterium mj15*	–	–	–	–	–	–	–
–	[c]*Bacterium bx4*	–	–	–	–	–	–	–
–	Uncultured bacterium	–	–	–	–	–	–	–

Polyherbal medicines were collected from the following: *KWTa* King Williamstown site A, *KWTb* King Williamstown site B, *KWTc* King Williamstown site C, *AL* Alice, *EL* East London, *FB* Fort Beaufort, *HBft* Hogsback first treatment, *HBst* Hogsback second treatment, *HBtt* Hogsback third treatment

– absent, *pathogenic to human, *a* Enterobacteriaceae, *b* Bacilliaceae, *n* non-pathogenic, *c* unclassified bacteria, *e* Clostridiaceae, *f* Oxalobacteraceae, *g* Mycobacteriaceae, *h* Paenibacillaceae, *i* Streptomycetaceae, *j* Eubacteriaceae, *k* Lactobacillaceae, *l* Leuconostocaceae, *m* Burkholderiaceae, *p* Pseudomonadaceae

is an emerging human food-borne pathogen. *B. cereus* has been reported to be associated with severe local and systemic human infections such as endophthalmitis, pneumonia, lung infections, bloody diarrhoea, gastroenteritis and meningitis, posing a serious public health problem [40, 41]. The pathogenicity of *B. cereus* depends on its ability to colonise, persist and subsequently invade the host tissues [42]. The ability of this bacterium to produce emetic toxins has been also associated with gastro- and non-gastrointestinal infections [43].

B. anthracis is an obligate pathogen that infects many vertebrates. It is the causative agent of anthrax. Anthrax is an infectious disease that can infect humans in three different ways, namely cutaneous anthrax, inhalation anthrax and gastrointestinal anthrax [44]. The presence of this bacterium in the remedies could be due to the availability of its endospores in the soil where the herb ingredients are harvested. The presence of *Bacillus* sp. in these remedies poses great risks to the consumers.

Klebsiella species are Gram-negative, non-motile and usually encapsulated rod-shaped bacteria [45]. Species of this genus have been increasingly associated with hospital infections [46]. They are common pathogens of nosocomial pneumonia, septicaemia, urinary tract infection, wound infections, intensive care unit infections and neonatal septicaemias [45]. *K. pneumoniae*, *K. oxytoca* and *K. variicola* were detected in the remedies. *K. pneumoniae* is the most pathogenic to humans. Infections with this bacterium are usually hospital-acquired. These include urinary tract infection, pneumonia, intra-abdominal infection, bloodstream infection, meningitis and pyogenic liver abscess [47]. *K. pneumoniae* has been reported to posses several intrinsic and acquired mechanisms which make it resistant to several antimicrobial agents [48, 49].

S. enterica was identified in KWTb remedy. It is a rod-shaped, Gram-negative, flagellated facultative anaerobe. The bacterium is a medically important pathogen of both humans and animals. The presence of this bacterium in this remedy is of concern because *S. enterica* is known to cause diseases such as gastroenteritis, septicaemia and enteric fever clinically [50]. Like many other infectious diseases, the infection severity of *Salmonella* may vary depending on the resistance of individual to the pathogen, the immune system and the virulence strain [50].

Enterobacter species are rod-shaped, non-spore-forming, facultative anaerobes and Gram-negative bacilli bacteria. Several strains of these bacteria are increasingly been identified as nosocomial pathogens causing infections in hosts with impaired immunity [51]. They have been reported to cause 5 % of hospital-acquired septicemias, 5 % of nosocomial pneumonias, 4 % of nosocomial urinary tract infections and 10 % of postsurgical peritonitis cases [52]. Skin and soft tissue infection, endocarditis, intra-abdominal infection, septic arthritis, osteomyelitis and ophthalmic infection have been associated with these bacteria. *E. cloacae* is ubiquitous in nature, although generally not known to be an enteric pathogen; it is an opportunistic pathogen in humans [53, 54]. A wide spectrum of infections such as the urinary tract, lower respiratory tract, skin and soft tissue, biliary tract, wounds, intravenous catheters and the central nervous system have been associated with *Enterobacter cloacae* [55].

None of the analysed marketed polyherbal remedies had any form of food-based tests carried out on them since they are locally made medicines. This may probably account for the high discovery of bacterial population in the remedies. The results from different findings conducted on microbial quality of traditional herbal medicines have been alarming revealing the presence of pathogenic bacteria and other contaminants in the herbal therapies [13–15, 17, 56]. Several fatal infectious outbreaks have been associated with the use of heavily contaminated raw materials of natural origin with pathogens; thus, great efforts are necessary to guarantee constant and adequate quality [57].

The prevalence of the members of Enterobacteriaceae in all the herbal remedies was recorded (Fig. 3.2). Enterobacteria are found in nature and are often thought as indicating faecal contamination. Thus, their presence could be regarded as an index of the degree of contamination, which may indicate possible presence of harmful or disease causing organisms [58–60]. Also, higher numbers of spore-forming bacteria such as *Bacillus* sp. and a few numbers of *Clostridium* sp. found in the remedies could be due to the fact that some of these organisms produce spores which are resistant to harsh processing, elevated heat and dry conditions, thereby able to survive for a very long time on the products in dormant states [26]. Likewise, the members of Paenibacillaceae which are Gram-positive and are aerobic bacteria that are related to *Bacilli* were found in the remedies. Until recently, these organisms were not known to cause human disease, but now, there are several reports of human infections caused by some members of this genus [61].

Conclusions

The findings of this study revealed different bacteria populations in the polyherbal medicines used for the treatment of tuberculosis in the Eastern Cape Province of South Africa; this is a cause for concern. Since there are no legislative criteria governing the microbial quality of these therapies in South Africa, there is an urgent need for the Government to take adequate control measures to set specific standards for quality of these medicines, considering the fact that these medicines are being taking by immunocompromised individuals. Also, there is

a need to educate the public as well as the traditional healers in the Province, that proper hygienic condition should be maintained in all preparation processes starting from plant collection, processing, packaging and storage, as well as emphasizing on the implications of non-compliance to hygienic conditions to both the consumers and the traditional healers.

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Authors' contributions
EB carried out the laboratory work and drafted the manuscript. AM participated in the coordination and assisted in the drafting of the manuscript. AJ conceived the study, participated in its design and coordination and revised the manuscript for intellectual content. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
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CHAPTER FOUR

Fungal Metagenomes in Polyherbal Medicines used for the Treatment of Tuberculosis

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CHAPTER FOUR

TABLE OF CONTENT

Chapters **Page No**

Fungal Metagenomes in Polyherbal Medicines used for the Treatment of Tuberculosis

Abstract

The traditional systems of medicine have significantly become more accepted in the developed and developing countries due to their curative property, less toxicity and minimal side effects. However, several studies have shown that they are associated with microbial contaminants. This study aimed at identifying fungi in nine polyherbal medicines used for the treatment of tuberculosis in Eastern Cape Province, South Africa. Sequences of fungi DNA that encodes internal transcribed spacer (ITS) region were retrieved from the remedies. The ITS region of the fungal rRNA operon was amplified using ITS1 and ITS4 primers. The amplicons were visualized on agarose gel electrophoresis, followed by end repair and adaptor ligation. They were further purified and quantified using Library Preparation kit NEBNext® UltraT DNA Library Prep Kit for Illumina and run on Illumina's MiSeq platform. The study revealed that the polyherbal medicines are contaminated with fungi species. The predominant mycoflora obtained belongs to different genera or species of fungi. They include *Aspergillus, Penicillium, Alternaria, Candida, Ramularia, Cladosporium* and *Malassezia* among others. Some of these organisms are capable of causing infections in immunocompromised patients. Thus, the study identified various fungal contaminants in polyherbal remedies sold to tuberculosis patients in five communities in the Eastern Cape Province of South Africa.

Key words: Fungi, ITS region, Polyherbal medicines, Public health, Tuberculosis

INTRODUCTION

The use of polyherbal formulations for therapeutic purposes has significantly increased in the developed and developing countries because of their curative property, less toxicity and minimal side effects (Binu, 2008; Devi et al., 2010). These benefits have made the usage of herbal medicines to be intertwined with that of modern medicine, thus, increasing the global market by 7% annually (Dubey et al., 2008). Traditional healers, especially those in low-income countries make use of various herbal preparations for the treatment and management of ailments such as wound infection, skin diseases, diabetes, diarrhoea, urinary tract infections, stomach illnesses and tuberculosis (Louw et al., 2002; Buwa and Afolayan, 2009).

Tuberculosis (TB), caused by a bacterium called *Mycobacterium tuberculosis* is the major killer among the infectious diseases and it is the ninth-leading cause of death worldwide. An estimate of 10.4 million new TB cases was reported in 2016, of this population, 1.7 million deaths including human immunodeficiency virus-TB co-infected individual were recorded (WHO, 2017). Seven countries have been implicated to have the highest burden of TB cases, thus responsible for 64% of the world TB burden. These countries include India, Indonesia, China, Philippines, Pakistan, Nigeria and South Africa (WHO, 2017). According to the Statistics for South Africa, TB is a significant public health challenge accounting for 7.2% of all death in 2016 followed by diabetes mellitus (SSA, 2017). The report of WHO (2016) gave an estimated incidence of 454,000 cases of active TB in South Africa; this implies that, about 0.8% of the 54 million South Africa populations develop active TB diseases (WHO, 2016).

In South Africa, about three million people make use of herbal remedies for their health care purposes especially for the treatment of infection disease such as TB (Louw et al., 2002; Elujoba et al., 2005). Despite the increasing use of herbal preparations and the global expansion of the market, safety is of great concern. Some studies have revealed that due to

unscientific mode of harvesting, drying, transportation, cleaning and handling of these herbal preparations, the raw plants prone to infestations and exposed them to different kinds of microbial contaminants (Stevic et al., 2012). The dominating contaminants are the bacterial endospores and fungal spores with the remaining are heavy metals and viruses originating from the soil (Adeleye et al., 2005; Kaume et al., 2012; Ting et al., 2013; Noor et al., 2014).

A few surveillance studies (Czech et al., 2001; Tassaneeyakul et al., 2004; Okunlola et al., 2007; Kulshrestha et al., 2008) have been conducted and have shown the presence of microbial contaminants in herbal preparations. Walther et al. (2016) investigated 109 traditional liquid herbal medicinal products in Mwanza city, the findings revealed that 81.7% of the samples were contaminated with fecal coliforms. The microbial quality of some oral liquid herbal medicines marketed in Ile-Ife conducted showed that 90% of the samples carried microbial loads beyond officially permissible limits (Igbeneghu and Lamikanra, 2016). Also, the quality control of hypoglycemic herbal preparations in Nairobi investigated have shown that the preparations are contaminated with both bacterial and fungal contaminants (Chege et al., 2015).

Siakrwar et al. (2014) isolated and identified a wide spectrum of fungi including *Aspergillus, Penicillium, Alternaria, Rhizopus* and *Syncephalastrum* species 15 medicinal plants. Toma and Abdulla, (2013) found that most of the fungal species detected in different types of spices and medicinal plants were *Aspergillus* spp. and *Penicillium* spp. while *Stachybotrys* sp., *Syncephalastrum racemocum, Uocladium botrytis, Alternaria alternata, Cladosporium lignicolum* and *Gliocladium catenulatum* were less frequently detected. Quality assessment of aqueous herbal/medicinal products has shown that the most abundant fungi were from *Cladosporium herbarum.* This was then followed by *Aspergillus spp., Saccharomyces kluyverii, Rhodotorulla minuta, Candida membranifasciens* and *Sporobolomyces salmonicolor* (Osei-Adjei et al., 2013).

The presence of numerous fungal species in herbal preparations can be harmful to consumers. Thus, in other to safe guard the health of the consumers, this study aimed at identifying the presence of different fungi in polyherbal medicines used for the treatment of tuberculosis in Amathole District Municipality, Eastern Cape Province, South Africa.

MATERIALS AND METHODS

Sample collection

This study is a secondary data analysis of the first authors' research project "Ethno-medicinal documentation of polyherbal medicines used for the treatment of tuberculosis in Amathole District Municipality of the Eastern Cape Province, South Africa" where information about the herbs used for the preparation of this remedies are revealed (Famewo et al., 2017). A total of nine different polyherbal medicines were purchased from the traditional herbal healers in five different communities namely; East London (EL), King Williams Town (KWT), Hogsback (HB), Alice (AL) and Fort Beaufort (FB) as shown in Figure 4.1. These remedies were liquid preparations and each of them was already homogenized and packaged in a 2 liters container by the herbal healers. Each remedy was labelled and coded according to the place of collection. The number of remedies obtained in this study was due to the fact that only a few traditional healers treat and sell the remedies for the treatment of tuberculosis. They claim to have acquired the knowledge from their ancestors, and this knowledge is being transferred from one generation to another. The samples were transported to Medicinal Plants and Economic Development (MPED) Research Centre Microbiology Laboratory for analysis.

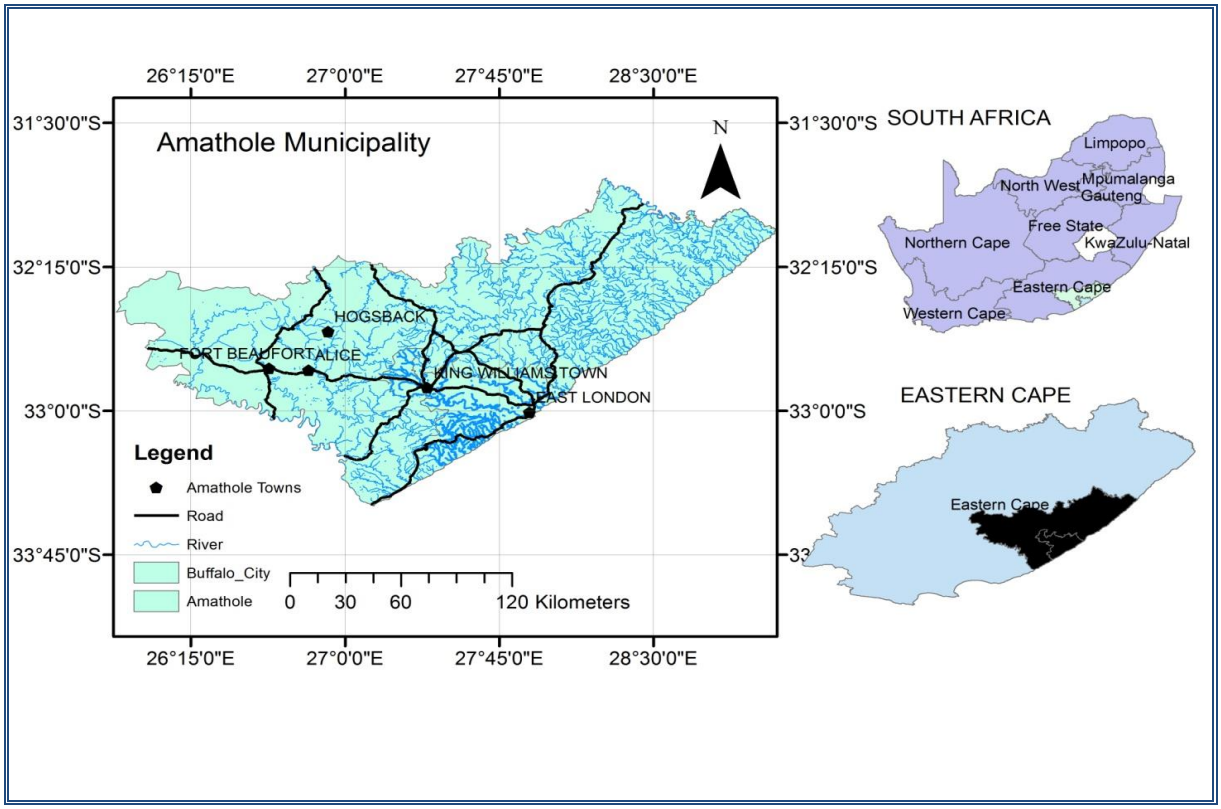

Figure 4.1. Map of Amathole District Municipality (Famewo et al., 2016)

DNA extraction

A modified method of Dei-Tutuwa et al. (2014) was used for the fungal DNA extraction. One ml of each sample was pipetted into Eppendorf tubes and centrifuged at 12500 rpm for 10 mins, the supernatant was discarded and the cell pellets was collected. The total fungal DNA was extracted using ZR Fungal/Bacterial DNA MiniPrep^{TM} Kit (Zymo Research, USA) according to the manufacturer instructions.

Amplification of fungal DNA using polymerase chain reaction (PCR)

The assay was conducted using the internal transcribed spacer (ITS) region of the fungal genome which is highly variable among species or even populations of the same species (Schoch et al., 2012). This region lies between the 18S small subunit (SSU) and 28S large subunit (LSU) ribosomal RNA (rRNA) genes, which also contains two non-coding spacer regions (ITS-A and ITS-B) separated by the 5.8S rRNA gene. The total genomic fungal DNA

was amplified using forward ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). PCR reaction was carried out in a final volume of 25 µL consisting England Biolabs, USA 5 µL template DNA, 12.5 µL of Q5 ® Hot start High-Fidelity 2X PCR Master Mix (New), 1 µL of 10 pmol each of the oligonucleotide primers (Inqaba Biotech, SA) and 5.5 µL of nuclease free water). Reactions were performed in the thermocycler (Bio-Rad Mycycler, USA) under the following conditions: initial denaturation at 95 °C for 2 min, followed by 30 cycles at 95 °C for 20 sec, 55 °C for 30 sec, and 72 °C for 30 sec, and a final elongation at 72 °C for 5 min (Kuo et al. 2005). In order to confirm the PCR products size, 5 µl of the amplicons was analyzed by gel electrophoresis in 1% agarose (Merck, SA) stained with 3 µl ethidium bromide (Sigma-Aldrich, USA). A 100 bp DNA ladder (Thermo Scientific, (EU) Lithuania was included for band size estimation purposes. All gels were run in 0.5X TBE buffer at 95 V for 1 h and visualized by UV trans-illumination (Alliance 4.7, France).

Purification of amplicons and sequencing

The amplicons were purified using the Agencourt® Ampure® XP bead protocol (Beckman Coulter, USA). The amplicon libraries were purified using the Agencourt® Ampure® XP bead protocol (Beckman Coulter, USA). Library concentration was measured using Nebnext Library quant kit (New England Biolabs, USA) and quality validated using Agilent 2100 Bioanalyser (Agilent Technologies, USA). The samples were pooled in equimolar concentrations and diluted to 4nM based on library concentrations and calculated amplicon sizes. The library pool was sequenced on a MiSeq™ (Illumina, USA) using a MiSeq™ Reagent kit V3 600 cycles PE (Illumina, USA). The final pooled library was at 10pM with 20% PhiX as control amplicon sequencing protocol. Each sample was sequenced in the sense and antisense directions using ITS1 and ITS4 primers (White et al., 1990; Kozich et al., 2013).

Data analysis

The relative frequency of different fungal phylum in each of the remedy, percentage occurrence of each fungal family and the abundance of fungal families was calculated according to Girridher and Ready (1997).

% of frequency = Number of observation in which a species appeared X100

Total number of observation

RESULTS

Distribution of different fungal phylum in each of the polyherbal remedies

The mycological analysis of the nine polyherbal remedies revealed that all the herbal preparations are contaminated with different fungal phylum and species. The predominant fungal phylum identified in all the herbal remedies was *Ascomycota* followed by *Basidiomycota*. The presence of 5% Glomeromycota was identified in KWTb and HBss remedies while 5% Zygomycota was found in KWTc remedy (Figure 4.2).

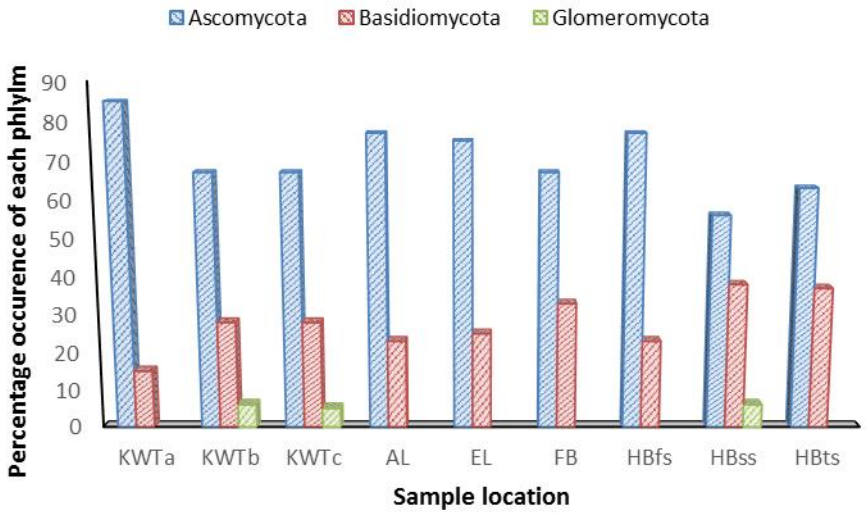

Figure 4.2. Relative frequencies of contaminating organisms in each of the polyherbal remedy

Distribution of different fungal families in all the polyherbal remedies

A total of 43 different fungal families were identified in all the nine polyherbal remedies. Members of the families Davidiellaceae, Mycosphaerellaceae, Trichocomaceae, Pleosporaceae and Saccharomycetaceae were identified in all the remedies (Figure 4.3). This was followed by Debaryomycetaceae, Malasseziaceae, Dothioraceae, Herpotrichiellaceae and Tremellaceae.

The abundance of each fungal family in all the remedies is represented in Figure 4.4 below. Members of the family Trichocomaceae were the most abundant followed by Mycosphaerellaceae, Pleosporaceae, Saccharomycetaceae, Tremellaceae, Davidiellaceae, Malasseziaceae and Sporidiobolaceae.

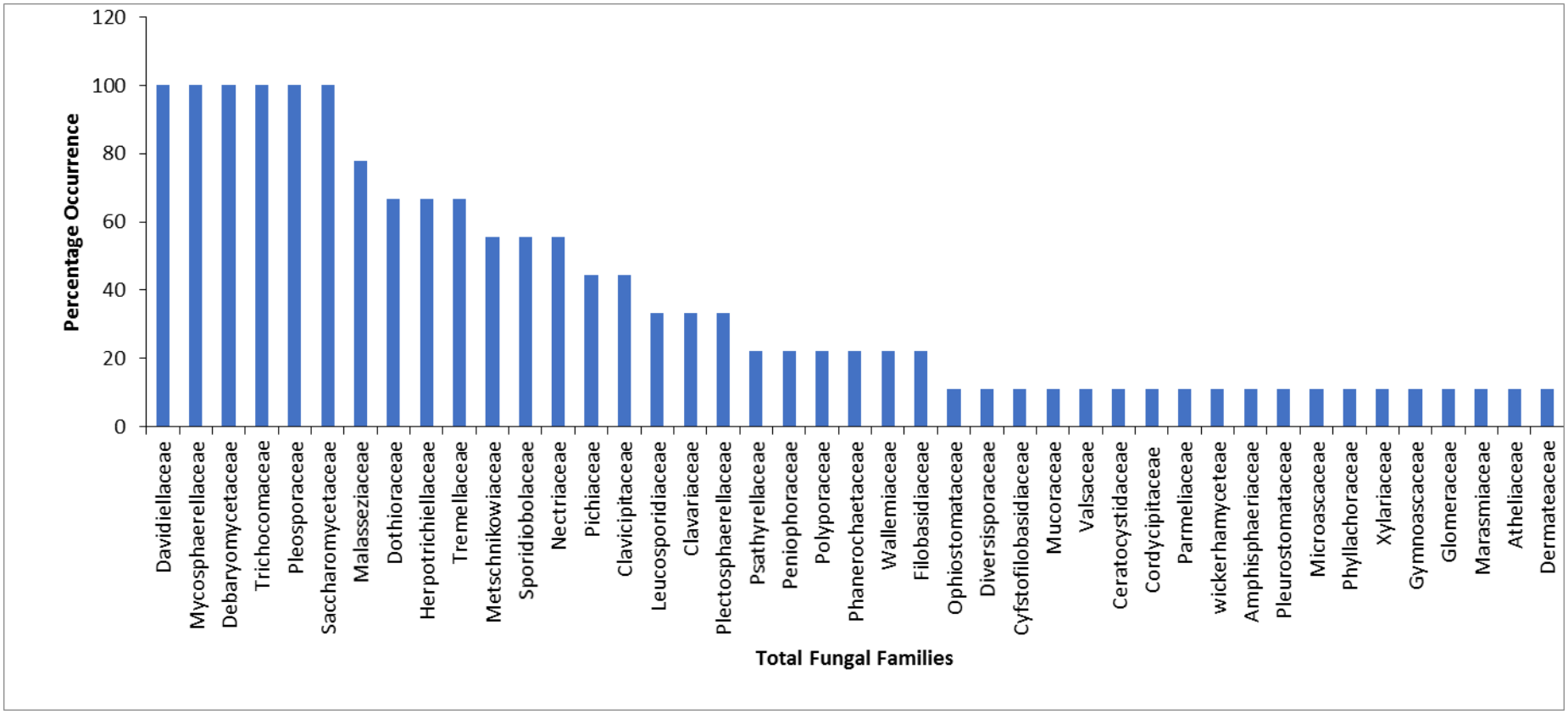

Figure 4.3. Percentage occurrence of each fungal family identified in the polyherbal remedies

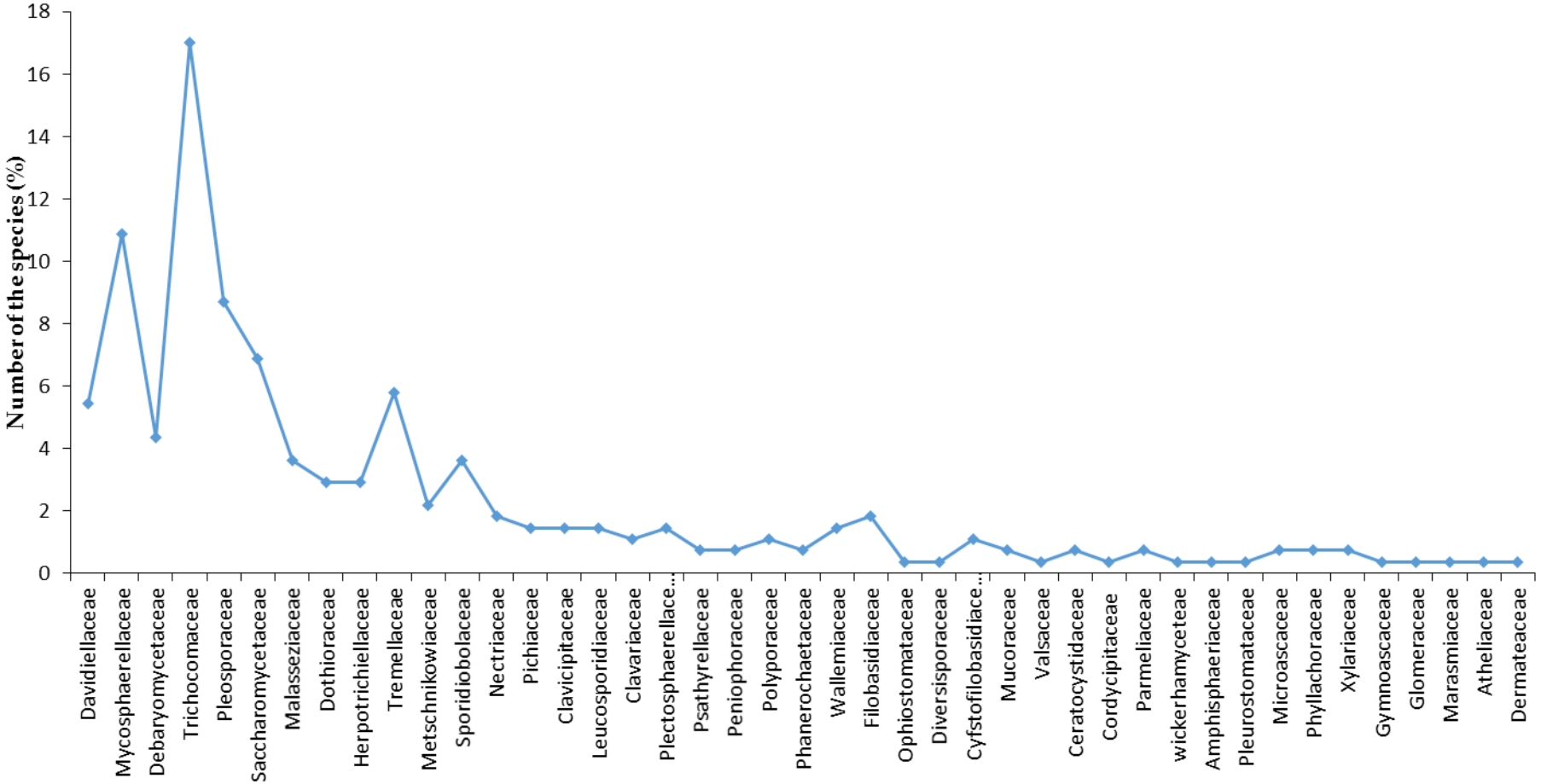

Figure 4.4. The abundance of fungal families in all the polyherbal remedies

Distribution of different fungal genera and species in each of the polyherbal remedies

By analyzing each of the herbal preparation (supplementary material), KWTa remedy was mainly contaminated with species of *Debaryomyces*, *Penicillium* and *Ramularia*. However, *Rhodotorula*, *Cladosporium*, *Ramularia*, *Candida* and *Malassezia* species were found in KWTb remedy. The remedy from KWTc was contaminated with *Ramularia, Candida, Cryptococcus, Rhodotorula, Cystofilobasidium* and *Mucor* species.

While AL remedy was mostly dominated with *Ramularia*, *Aspergillus, Penicillium, Candida, Rhinocladiella* and *Parmotrema* species; *Cladosporium*, *Ramularia*, *Alternaria*, *Candida* and *Malassezia* were identified in EL remedy. The presence of *Cladosporium*, *Ramularia, Aspergillus, Penicillium, Alternaria* and *Candida* were detected in FB remedy.

While species of *Cladosporium*, *Ramularia*, *Aspergillus* and *Penicillium* dominated the mycofloral of HBfs remedy, HBss remedy was contaminated with *Cladosporium*, *Ramularia* and *Aspergillus*. However, *Cladosporium, Ramularia*, *Aspergillus*, *Penicillium*, *Alternaria* and *Wallemia* species were detected in HBts remedy.

Across all the herbal remedies, the most predominant mycoflora obtained was distributed in four different genera which comprised of *Candida*, *Cladosporium*, *Ramularia* and *Alternaria*. This was followed by *Aspergillus* and *Penicillium* which were found in seven and eight remedies, respectively (supplementary material).

DISCUSSION

The use of polyherbal medicines for the treatment and management of various illnesses is part of the health-care culture in South Africa. These remedies are prepared locally by traditional healers by combining two or more parts of medicinal plants such as the root, leaf, stem, flower and seed. The results obtained in the present study revealed the presence of different fungi contaminants particularly moulds and yeast in the polyherbal remedies, which are used for the

treatment of TB. The presences of these organisms may pose potential health risks to tuberculosis patients considering their immunocompromised status. The high fungal population in each of the remedies is an indication of low environmental sanitation and unhygienic standard of processing these herbal medicines. All the remedies were stored at room temperature in the shops and there were no expiry date written on them, thus, the storage condition could have encouraged the growth of these fungal species.

Many of the fungal species identified are naturally inhabitant of the soil and some are plant pathogens. According to Sharma (2001), fungal contamination of herbal preparations mainly occurs during a slow drying process. Inadequate drying or postharvest storage of the herbs under a high relative humidity and favourable temperature promotes the growth of these microbes (Sharma, 2001). Also, the unscientific methods of collection, unsuitable transportation and prolonged storage of the plants and inadequate hygiene of the handler could trigger the growth of organisms in herbal medicines (Sago et al., 2009; Stević et al., 2012).

The presence mould such as *Aspergillus* and *Penicillium* species in seven and eight polyherbal remedies, respectively, could be attributed to the growth of these organisms in the herbs before the medicinal plants were completely dried (Stevic et al., 2012). Both species of *Aspergillus* and *Penicillium* have been associated with food poisoning and may cause infections in an immunosuppressed individuals (Lin and Teutsch, 2001; Bateman et al., 2002). The results of this study were in well agreement with those found by Tournas and Katsoudas (2008). The study examined the microbiological quality of various medicinal herbal teas. The findings revealed that the most common fungal contaminants in the herbal teas were *Aspergillus niger*, *Penicillium* spp., *Eurotium rubrum, E. chevalieri, A. flavus*, *Fusarium spp.*, *Alternaria alternata* and yeasts. Also, a South African study has reported contamination of herbal products with bacteria as well as fungi such as Penicillium and Aspergillus (Govender et al., 2006). Examination of pathogenic microorganisms in medicinal herbal drugs has

equally shown that the most abundant fungi species were from *Fusarium, Aspergillus* and *Alternaria* according to Stevic et al. (2012). In addition, the fungal contamination of powdered herbal medicinal preparations sold in some parts of Nigeria was evaluated, the results showed that all of the herbal preparations had the presence of fungal contaminants with predominance of *Aspergillus* spp. and *Penicillium* spp., while *Mucor* spp., *Candida* spp. and *Trichosporium* spp., were also present (Anyanwu, 2010). Similar to this study, is the findings of Zheng et al. (2017) which revealed that the surface of medicinal herbs are predominantly contaminated with species of *Aspergillus* and *Penicillium*.

Aspergillus is a group of moulds found in natural environment, it is an airborne fungus capable of causing Aspergillosis. Species of this genus are highly aerobic, possesses the ability to grow where high osmotic pressure exist and are found in oxygen rich environment. They are capable of growing at low water content; thus, to avoid their growth, quick drying of the herbs are highly important (Stevic et al., 2012). There species are detectable in the ground, air and in plants. Aspergillus does not normally cause infection except in an immunocompromised individuals such as leukaemia, asthma, HIV/AIDS and in people with damaged lungs due to TB infection, thus causing severe pulmonary disease (WHO, 2011). According to WHO Bulleting, about one-third of TB patients develop cavities in their lungs, thus making them vulnerable to the infection (WHO, 2011). The presence of this Aspergillus in the remedies could be detrimental to the health of TB-patients considering their immunosuppressed status.

Species of *Fusarium* were also detected in four of the remedies. In a study carried out by Stevic et al. (2012), *Fusarium* was observed as the most dominant genus in most of the herbal drugs tested. The spores of this organism can survive drying conditions and remain dormant for several months. They are found abundantly in the soil and many of them are important plant pathogens causing various diseases such as crown rot, head blight and scab on

cereal grains (Nelson et al., 1994). Some strains such as *Fusarium fumonisins* and *F. trichothecenes* are toxins producers; however, they were not identified in these remedies. Several species of *Fusarium* have emerged as important opportunistic pathogens in humans causing a broad spectrum of infections such as hyalohyphomycosis, mycotic keratitis, onychomycosis, pneumonia, disseminated infections and sinusitis mostly in immunocompromised patients (Makowsky et al., 2005; Guarro, 2013). Species of *Fusarium* identified in this study are *F. oxysporum, F. verticillioides* and *F. delphinoides*. Many studies have reported cases of Fusariosis in patients with acute myeloid and lymphoblastic leukemia (Jossi et al., 2010) however; there is dearth information on Fusarium infections associated with tuberculosis patients. Considering the immune-deficiency of the patients, the consumption of these remedies over a long period and prolong storage should be avoided in order to prevent the production of mycotoxins in the remedies.

Alternaria, Candida and *Ramularia* species were also found in all the tested remedies. *Alternaria* species are plant pathogens and field mycotoxin-producing moulds (Stevic et al., 2014). They are capable of producing tenuazonic acid and other toxic metabolites which may be associated with diseases in humans or animals. These organisms have been reported as causative agents of subcutaneous phaeohyphomycosis, mycotic keratitis, hypersensitivity pneumonitis and extrinsic asthma (Crissey et al., 1995). They equally cause infections such as allergic bronchopulmonary mycosis and pheohyphomycotic lumbar spondylodiscitis in immunocompromised individuals. Therefore, it is important to maintain these remedies at the temperature or condition that would not support the growth this organism.

Candida species are yeasts and the most common cause of global fungal infections called Candidiasis (Manolakaki et al., 2010). They are commensals that colonize the skin, gastrointestinal and reproductive tracts. The species of *Candida* identified in this study are non-pathogenic strains but are capable of causing infections in immunocompromised patients.

Species of these organisms have been reported as emerging pathogenic fungal in patients with pulmonary tuberculosis (Kali et al., 2013; Ndukwu et al., 2016). Since *Candida* species can be found on the skin, coupled with the unsterile mode of preparation of these remedies, it could be said that this organism was introduced into the remedies by the handler during the process of preparation. The presence of these fungal contaminants in the polyherbal remedies can reduce or inactivate the therapeutic activity of the remedies and possess the potential to adversely affect the tuberculosis patients.

Conclusion

The findings of this study indicated that polyherbal medicines marketed in the study area are contaminated with fungi contaminants. Some of these organisms are capable of causing infections in immunocompromised patients while others are plant pathogens. The contamination of these polyherbal preparations could have resulted from contaminated soil, plants, inadequate drying, unhygienic mode of preparation and possibly prolonged storage. This could probably be a potential health risk to consumers. It is therefore suggested that quality-control measures and safe handling practices be established for medicinal herbs in the Province.

Acknowledgements

The work was supported by National Research Foundation of South Africa. The authors also acknowledge the traditional healers for selling the polyherbal remedies to us.

Conflict of Interests

The authors declared no conflict of interests.

Table 1: Supplementary Material: Identified fungal families, genera and species in all the polyherbal

Families	**KWTa**	**KWTb**	**KWTc**	**AL**	**EL**	**FB**	**HBfs**	**HBss**	**HBts**
Davidiellaceae	*Cladosporium tenuissimum*	Uncultured *cladosporium*	*Uncultured cladosporium*	*Cladosporium cladosporioides*	*Cladosporium cladosporioides*	*Cladosporium cladosporioides*	*Cladosporium cladosporioides*	*Davidiella tassiana*	*Cladosporium sp.*
		Cladosporium cladosporioide			*Cladosporium sp.*	*Cladosporium sphaerospermum*		*Cladosporium cladosporioides*	*Davidiella tassiana*
		Cladosporium sphaerospermum							
Mycosphaerellaceae	*Ramularia eucalypti*	*Ramularia eucalypti*	*Ramularia eucalypti*	*Ramularia mali*	*Ramularia eucalypti*	*Ramularia mali*	*Cladosporium herbarum*	*Ramularia eucalypti*	*Ramularia eucalypti*
	Ramularia mali	*Ramularia mali*	*Ramularia mali*	*Ramularia eucalypti*	*Ramularia mali*	*Ramularia eucalypti*	*Cercospora sojina*	*Ramularia mali*	*Ramularia mali*
		Cladosporium herbarum		*Cladosporium herbarum*	*Ramularia vizellae*	*Cladosporium herbarum*	*Ramularia mali*	*Cladosporium herbarum*	*Cladosporium herbarum*
				Ramularia vizellae	*Cercospora sp.*		*Ramularia eucalypti*		*Cladosporium sphaerospermum*
									Cercospora sojina
Debaryomycetaceae	*Debaryomyces hansenii*	*Meyerozyma guilliermondii*	*Meyerozyma guilliermondii*	*Meyerozyma guilliermondii*	*Debaryomyces hansenii*	*Meyerozyma guilliermondii*	*Debaryomyces hansenii*	*Debaryomyces nepalensis*	*Meyerozyma guilliermondii*
	Meyerozyma guilliermondii		*Debaryomyces hansenii*						
	Debaryomyces macquariensis								
Trichocomaceae	*Aspergillus niger*	*Talaromyces amestolkiae*	*Aspergillus pseudoglaucus*	*Aspergillus pseudoglaucus*	*Penicillium sp.*	*Aspergillus steynii*	*Aspergillus pseudoglaucus*	*Aspergillus niger*	*Aspergillus pseudoglaucus*
	Penicillium commune		*penicillium brevicompactum*	*Aspergillus fumigatus*		*Aspergillus westerdijkiae*	*Talaromyces amestolkiae*	*Aspergillus pseudoglaucus*	*Penicillium chrysogenum*

	Penicillium crustosum		*Talaromyces amestolkiae*	*Aspergillus amstelodami*		*Aspergillus pseudoglaucus*	*Penicillium oxalicum*	*Talaromyces amestolkiae*	*Aspergillus ruber*
				Aspergillus ruber		*Talaromyces amestolkiae*	*Aspergillus ruber*	*Penicillium commune*	*Aspergillus amstelodami*
				Penicillium verrucosum		*Aspergillus amstelodami*	*Aspergillus amstelodami*	*Aspergillus ruber*	*Talaromyces amestolkiae*
				Penicillium chrysogenum		*Aspergillus proliferans*	*Penicillium commune*		*Penicillium brevicompactum*
				Penicillium brevicompactum		*Aspergillus versicolor*			*Penicillium steckii*
						Aspergillus ruber			*Aspergillus appendiculatus*
						Penicillium chrysogenum			*Aspergillus nomius*
						Penicillium brevicompactum			
						Aspergillus ostianus			
						Penicillium commune			
Pleosporaceae	*Alternaria alternata*	*Alternaria alternata*	*Alternaria alternata*	*Alternaria alternata*	*Stemphylium sp.*	*Alternaria sp.*	*Alternaria alternata*	*Epicoccum nigrum*	*Alternaria porri*
				Leptosphaerulina chartarum	*Alternaria alternata*	*Alternaria alternata*	*Stemphylium sp.*	*Alternaria alternata*	*Alternaria alternata*
				Phoma sp.	*Epicoccum nigrum*	*Alternaria tenuissima*	*Phoma sp.*	*Phoma plurivora*	
				Pyrenophora tritici-	*Alternaria japonica*	*Paraconiothyrium*			

				repentis		*sp.* *Bipolaris cynodontis*			
Saccharomycetaceae	*Candida intermedia*	*Candida oleophila* *Candida intermedia*	*Candida oleophila* *Candida railenensis*	*Candida parapsilosis* *Candida oleophila*	*Candida sp.* *Candida zeylanoides*	*Pichia kudriavzevii* *Candida intermedia* *Yamadazyma triangularis* *Candida etchellsii* *Candida oleophila*	*Candida etchellsii*	*Candida sp.* *Pichia fermentans*	*Candida orthopsilosis*
Malasseziaceae		*Malassezia globosa* *Malassezia restricta*		*Malassezia restricta*	*Malassezia globosa* *Malassezia sympodialis*	*Malassezia restricta*	*Malassezia restricta*	*Malassezia restricta* *Malassezia sympodialis*	*Malassezia restricta*
Dothioraceae	*Aureobasidium pullulans*	*Aureobasidium pullulans*	*Aureobasidium pullulans*		*Rhodotorula sp.* *Arxiella dolichandrae* *Aureobasidium pullulans*			*Aureobasidium pullulans*	*Aureobasidium pullulans*
Herpotrichiellaceae		*Rhinocladiella similis*	*Exophiala bergeri*	*Rhinocladiella similis* *Rhinocladiella atrovirens*	*Phialophora mustea*	*Rhinocladiella similis* *Phialophora mustea*			*Rhinocladiella similis*
Tremellaceae		*Cryptococcus amylolentus*	*Cryptococcus laurentii*			*Cryptococcus heimaeyensis*	*Cryptococcus albidosimilis*	*Kwoniella mangrovensis*	*Cryptococcus zeae*

		Kwoniella heveanensis	*Cryptococcus albidosimilis* *Cryptococcus adeliensis*			*Cryptococcus albidosimilis*	*Cryptococcus sp.*	*Cryptococcus albidosimilis* *Bullera dendrophila*	*Kwoniella mangrovensis* *Bullera dendrophila*
Metschnikowiaceae		*Clavispora lusitaniae*	*Clavispora lusitaniae* *Metschnikowia pulcherrima*	*Clavispora lusitaniae*		*Metschnikowia corniflorae*		*Clavispora lusitaniae*	*Cryptococcus albidosimilis*
Sporidiobolaceae		*Sporidiobolales* sp. *Rhodotorula fujisanensis* *Rhodotorula mucilaginosa* *Rhodosporidium babjevae*	*Rhodotorula mucilaginosa* *Rhodotorula colostri* *Rhodotorula fujisanensis*	*Rhodotorula mucilaginosa*	*Rhodotorula mucilaginosa*			*Rhodotorula mucilaginosa*	
Nectriaceae	*Fusarium oxysporum*	*Fusarium delphinoides*	*Fusarium verticillioides*	*Gibberella pulicaris*			*Fusarium oxysporum*		
Pichiaceae	*Pichia fermentans*	*Issatchenkia hanoiensis*	*Issatchenkia hanoiensis*						*Yamadazyma triangularis*
Clavicipitaceae	*Metarhizium flavoviride*	*Metarhizium flavoviride*				*Metarhizium flavoviride*			*Metarhizium flavoviride*
Leucosporidiaceae	*Leucosporidium scottii*	*Uncultured leucosporidium* *Leucosporidium scottii*	*Uncultured leucosporidium*						

Clavariaceae	*Uncultured clavulinopsis*		*Uncultured clavulinopsis*		*Uncultured clavulinopsis*		
Plectosphaerellaceae					*Plectosphaerella cucumerina* *Gibellulopsis nigrescens*	*Uncultured gibellulopsis*	*Gibellulopsis nigrescens*
Psathyrellaceae		*Coprinopsis episcopalis*				*Coprinellus sp.*	
Peniophoraceae				*Peniophora sp.*	*Peniophora sp.*	*Peniophora sp.* *Peniophora incarnata*	
Polyporaceae				*Trametes sanguinea* *Pycnoporus coccineus*			*Daedaleopsis sp.*
Phanerochaetaceae					*Phanerochaete sordida*		*Phanerochaete sordida*
Wallemiaceae					*Wallemia sebi*		*Wallemia mellicola* *Wallemia sebi* *Wallemia muriae*
Filobasidiaceae						*Filobasidium elegans* *Filobasidium floriforme*	*Filobasidium elegans* *Filobasidium floriforme*

Ophiostomataceae	*Ophiostoma ips*			*Cystofilobasidium capitatum*
Diversisporaceae	*Uncultured diversispora*			
Cyfstofilobasidiaceae		*Cystofilobasidium ferigula* *Guehomyces pollulans* *Cystofilobasidium capitatum*		
Mucoraceae		*Mucor circinelloides* *Mucor fragilis*		
Valsaceae		*Cytospora austromontana*		
Ceratocystidaceae			*Ceratocystis paradoxa* *Microascales sp.*	
Cordycipitaceae			*Lecanicillium psalliotae*	
Parmeliaceae			*Parmotrema austrosinense* *Parmotrema tinctorum*	
Wickerhamyceteae			*Wickerhamomyces anomalus*	

Amphisphaeriaceae	*Pestalotiopsis disseminata*			
Pleurostomataceae		*Pleurostomophora richardsiae*		
Microascaceae		*Sphaeronaemella fimicola* *Pseudallescheria boydii*		
Phyllachoraceae			*Uncultured phyllachora* *Phyllachorales sp.*	
Xylariaceae			*Sordariomycetes sp.* *Xylaria sp.*	
Gymnoascaceae			*Uncultured gymnoascus*	
Glomeraceae				*Uncultured glomus*
Marasmiaceae				*Marasmiellus violaceogriseus*
Atheliaceae				*Piloderma sp.*
Dermateaceae				*Gloeosporium sp.*

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CHAPTER FIVE

Anti-*Mycobacterium tuberculosis* activity of polyherbal medicines used for the treatment of tuberculosis in Eastern Cape, South Africa

CHAPTER FIVE

TABLE OF CONTENT

Anti-mycobacterium tuberculosis activity of polyherbal medicines used for the treatment of tuberculosis in Eastern Cape, South Africa.

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Abstract
Background: The emergence of drug-resistant strains of *Mycobacterium tuberculosis* has become a global public health problem. Polyherbal medicines offer great hope for developing alternative drugs for the treatment of tuberculosis.
Objective: To evaluate the anti-tubercular activity of polyherbal medicines used for the treatment of tuberculosis.
Methods: The remedies were screened against *Mycobacterium tuberculosis* H37Rv using Middlebrook 7H9 media and MGIT BACTEC 960 system. They were liquid preparations from King Williams Town site A (KWTa), King Williams Town site B (KWTb), King Williams Town site C (KWTc), Hogsback first site (HBfs), Hogsback second site (HBss), Hogsback third site (HBts), East London (EL), Alice (AL) and Fort Beaufort (FB).
Results: The susceptibility testing revealed that all the remedies contain anti-tubercular activity with KWTa, KWTb, KWTc, HBfs, HBts, AL and FB exhibiting more activity at a concentration below 25 µl/ml. Furthermore, MIC values exhibited inhibitory activity with the most active remedies from KWTa, HBfs and HBts at 1.562 µg/ml. However, isoniazid showed more inhibitory activity against *M. tuberculosis* at 0.05 µg/ml when compare to the polyherbal remedies.
Conclusion: This study has indicated that these remedies could be potential sources of new anti-mycobacterial agents against *M. tuberculosis*. However, the activity of these preparations and their active principles still require in vivo study in order to assess their future as new anti-tuberculosis agents.
Keywords: *Mycobacterium tuberculosis*; in vitro activity, polyherbal medicines, South Africa.
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Introduction
Mycobacterium tuberculosis, the leading causative agent of tuberculosis (TB) is responsible for the morbidity and mortality of a large population worldwide[1]. TB has a long co-evolutionary history with humans. It does not exhibit any symptom of disease except when impairment of immunity arises due to malnutrition, diabetes, malignancy and AIDS[2]; however, about 10% of healthy individuals

may develop active TB in their life time due to genetic factors. The ability of TB to resist drugs and the influence of HIV epidemic has made the disease remain a devastating global public health problem[3]. According to WHO[4], one-third of the world's population have been infected with *Mycobacterium tuberculosis* (MTB). In 2014, an estimated number of 9.6 million new TB infections were reported, of which 5.4 million were men; 3.2 million were women and 1.0 million children[3]. This disease is responsible for approximately two million deaths annually[5].

Some of the main obstacles to the global control of the disease are the HIV epidemic that has dramatically increased the risk of developing active TB, increasing emergence of multidrug resistant-TB (MDR-TB: resistance to isoniazid and rifampin) and refractory nature of latent TB treatment to conventional anti-TB drugs[6,7,8,9]. The situation is further exacerbated by the increasing development of extensively drug-resistant (resistant to MDR-TB, all fluoroquinolones and at least one of the second-line anti-TB injectable drugs including amikacin, kanamycin and/or capreomycin)[10,11]. According to the modes of action of these drugs, they can be grouped as cell wall inhibitors (isoniazid, ethambutol, ethionamide, cycloserine), nucleic acid synthesis inhibitors (rifampicin and quinolones), protein synthesis inhibitors (streptomycin, kanamycin) and inhibitors of membrane energy metabolism (pyrazinamide)[12,13,14]. For instance, Isoniazid (INH)is the most widely used treatment for TB and its latent infections[15]. This drug enters the cell as a pro-drug, which is activated by MTB catalase-peroxidase enzyme (KatG). The enzyme activates INH and facilitates its interaction with various toxic reactive species (oxides, hydroxyl radicals and organic moieties) in the bacterial cell[16], thereby, weakening the components of the cell wall and finally, the death of the bacteria[17]. INH targets inhA enzyme (enoylacyl carrier protein reductase), which is involved in the elongation of fatty acids in mycolic acid synthesis[18]. The replacement of an amino acid in the NADH binding site of inhA results into INH resistance, preventing the inhibition of mycolic acid biosynthesis[19]. INH-resistant strains often lose catalase and peroxidase activities due to KatG Ser315Thr mutation[20]. Resistance to INH can also occur through mutations in the promoter region of inhA, leading to over expression of inhA, or by mutations at the inhA active site, thereby lowering inhA affinity for INH[21].

Rifampicin (RIF) have been used as first-line drug in combination with other therapies for the treatment of TB infections. RIF is believed to inhibit bacterial DNA-dependent RNA polymerase[9]. This drug interferes with RNA synthesis by binding to the β subunit of *mycobacterial* RNA polymerase, which is encoded by rpoB, thereby killing the organism. Resistance to RIF arises due to missense mutations in the gene. Mtb resistance to RIF occurs at a frequency of 10−7 to 10−8 as a result of mutations in rpoB[22]. About 96% of all mutations are found in the 81-bp core region of the gene between codons 507 and 533, with the most common changes occurring in codons Ser531Leu,His526Tyr and Asp516Val[23].

Pyrazinamide (PZA) is another vital first-line drug used for the treatment of TB. It plays an important role in reducing the duration of TB treatment[24]. PZA is a pro-drug that requires conversion to its active form, pyrazinoic acid (POA) by the *mycobacterial* enzyme pyrazinamidase/nicotinamidase. The efflux system of the mycobacterial cell enables massive accumulation of POA in the bacterial cytoplasm, leading to disruption of the bacterial membrane potential[25,26]. The exact mechanism of PZA resistance remains unknown[9]. However, PZA resistance has been associated with defective pyrazinamidase/nicotinamidase activity which results from mutations that might occur at different regions (3-17, 61-85 and 132-142) of pyrazinamidase/ nicotinamidase[27].

Ethambutol (EMB) is a first-line drug used in combination with INH, RIF and PZA preventing the emergence of drug resistance *mycobacterium*. This drug interferes with the cell wall of MTB through a synthetic mechanism thereby inhibiting arabinosyl-transferase (embB), an enzyme involved in cell wall biosynthesis28. The enzyme has been proposed as the target of EMB in Mtb11. Mutation is the cause of EMB resistance and it occurs at a rate of approximately 1 in 107 organisms. It increases the production of arabinosyl-transferase, which overwhelms the inhibitory effects of EMB. Studies have revealed five mutations in codon 306 accounting 70–90% of all EMB resistant strains[29]. The resistance of Mtb to TB-drugs is mostly due to mutation which is a cause for concern. Therefore, it is important to search for new anti-*tuberculosis* agents, preferably those that can be readily and simply produced from medicinal plants.

It has been estimated that about 80% of South African population is infected with tuberculosis, with 88% highest prevalence of latent TB among the age group of 30-39 years old living in the rural settlements[30]. However, the strains of drug resistant tuberculosis have been on increase yearly in the country[31].

Polyherbal remedies have been used extensively for the treatment of various diseases for many centuries. They are mixtures of various herbs which contain multiple active constituents and act synergistically against infections[32]. Natural products and/or their semi-synthetic derivatives are important sources of new chemical compounds that might play an important role in the chemotherapy of tuberculosis[33]. Several studies on the use of polyherbal medicines have revealed that these therapies possess pharmacological functions. For instance, *Rajanyamalakadi*, a polyherbal preparation which contains three herbal ingredients has been proven to show significant anti-diabetic, hypolipidemic and anti-oxidant properties[34]. Also, Polyherbal health tonic tea used for the treatment of an array of diseases affecting humans and Sanjivani Vati used for the treatment of cough and cold have been shown to possess significant pharmacological activities[35,36]. Other Polyherbal remedies such as *Livina, Rhumapar* tablet, *Diakyur* and Sugar Remedy have been proven to contain pharmacological activities[37,38,39,40].

Many researchers have reported on the inhibitory properties of medicinal plants against *Mycobacterium tuberculosis* both in South Africa and in other countries[33,41,42] but there is a dearth of information on the inhibitory properties of polyherbal medicines against this organism. The aim of the present study therefore was to evaluate polyherbal remedies used for the treatment of TB for anti-*Mycobacterium tuberculosis* activities.

Materials and methods

Collection of polyherbal medicines

A total of nine polyherbal medicines evaluated in this study were purchased from herbal sellers in five communities namely; Alice, Fort Beaufort, Hogsback, King Williams Town and East London in Amathole District Municipality of the Eastern Cape Province, South Africa (Figure 1). Each remedy was labelled and coded according to the place of collection; viz: King Williams Town site A (KWTa), King Williams Town site B (KWTb), King Williams Town site C (KWTc), Hogsback first site (HBfs), Hogsback second site (HBss), Hogsback third site (HBts), East London (EL), Alice (AL) and Fort Beaufort (FB). The small number of remedies obtained in this study was due to the fact that only a few traditional healers treat and sell remedies for TB. They claim to have acquired the knowledge from their ancestors; and this knowledge is been transferred from one generation to another. The herbal ingredients present in each of the remedies are shown in Table 1. The remedies were already prepared with water by the herbal sellers into clean 2-litre containers. They were then transported to Medicinal Plants and Economic Development Research Centre, University of Fort Hare for analysis.

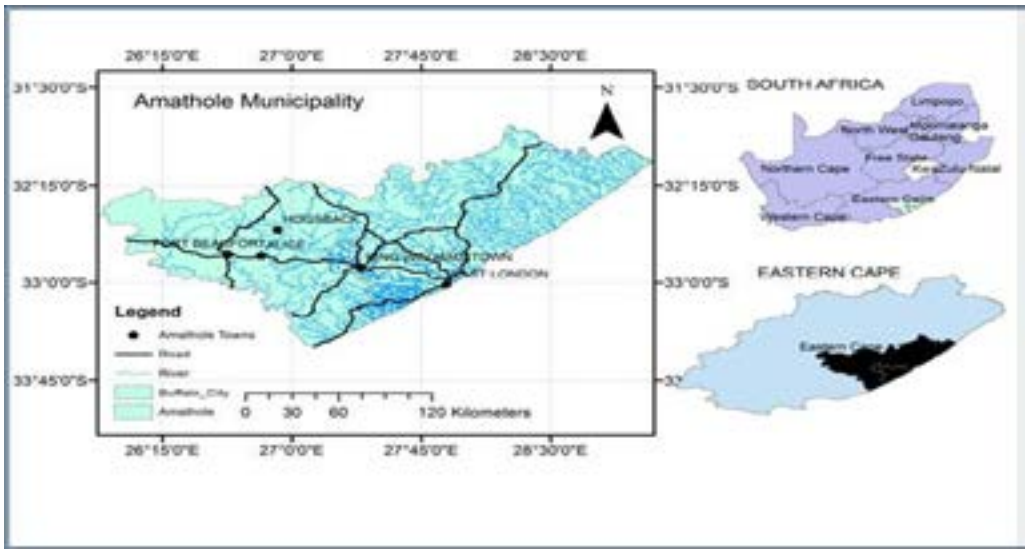

Figure 1: Map of Amathole District Municipality[43]

Table 1: Herbal ingredients present in each of the polyherbal medicines used for the treatment of tuberculosis in Amathole district municipality,

Name/code	Local name	Botanical name	Parts used
AL	Mountain garlic Mlomo mnandi Red carrot Inongwe Mnonono River pumpkin Herbal menthol leaf Herbal buchu water	*Allium sativum* (L.) *Glycyrrhiza glabra* (L.) *Daucus carota* (L.) *Hypoxis argentea* (Fiscand) *Strychnos decussata* (Pappe) Gilg *Gunnera perpensa* (L.) *Mentha piperita* (L.) *Agathosma betulina* (Berg)	Rhizome Root Root corms Bark Rhizome Leaf Leaf
EL	Inongwe Intelezi Ngcambumvuthuza Inqwebeba Iqwili	*Hypoxis argentea* (Fiscand) *Haworthia reinwardtii* (Haw) *Ranunculus multifidus* (Forssk) *Albuca flaccid* (Jacq.) *Alepidea amatymbica* (Eckl. & Zeyh.)	corms Leaf Root Leaf Rhizome
FB	Buchu leaf Mountain garlic Ginger Chilli pepper	*Agathosma betulina* (Berg) *Allium sativum* (L.) *Zingiber officinalis* (L.) *Capsicum annuum* (L.)	Leaf Rhizome Rhizome Fruit
KWTa	Maphipha Mnonono Ixonya Inongwe Sicimamlilo Iphuzi	*Rapanea melanophloeos* (L.) *Strychnos decussate* (Pappe) Gilg *Kniphofia drepanophylla* (Baker) *Hypoxis argentea* (Fiscand) *Pentanisia prunelloides* (Klotzsch) *Centella eriantha* (Rich.)	Bark Bark Root Corms Rhizome Rhizome
KWTb	Umdlavuza Mnonono Inceba emhlophe	*Lauridiatetragonia* (L.F.) *Strychnos decussate* (Pappe) Gilg *Hermannia* sp. (L.)	Root Bark Root
KWTc	Mnonono	*Strychnos decussate* (Pappe) Gilg	Bark
HBfs	Red carrot Mlungu mabele Calmoes Mountain garlic	*Daucus carota* (L.) *Zanthoxylum capense* (Thunb.) *Acorus calamus* (L.) *Allium sativum* (L.)	Root Bark Rhizome Rhizome
HBss	Buchu leaf Chilli pepper	*Agathosma betulina* (Berg) *Capsicum annuum* (L.)	Leaf Vegetable
HBts	Maphipha Red carrot Uroselina Mountain garlic	*Rapanea melanophloeos* (L) Mez *Daucus carota* (L.) *Cinnamomum camphora* (L.) J. Presl *Allium sativum* (L.)	Bark Root crop Bark Rhizome

Sample preparation

The already prepared water remedies were put in 2-liter containers. Each remedy was filtered with a Buchner funnel and Whatman No. 1 filter paper. The filtrate obtained was frozen at -40°C and freeze dried for 48h using a freeze dryer (Vir–Tis benchtop K, Vir–Tis Co., Gardiner, NY). The resulting sample was dissolved in 100% dimethylsulfoxide (DMSO) to a concentration of 50 mg/ml to make a stock solution[45].

Microbial strain and medium used for the assays

Reference MTB strain H37Rv (ATCC 25618) was used for the anti-*Mycobacterium tuberculosis* assay. It was obtained from American Type, MD, USA Culture Collection. Bacterial culture with DMSO (1.2%), isoniazid (INH) at MIC99 (0.05 μg/ml) and bacterial culture only were used as controls[46].

Bacterial culture and drug preparation

Suspensions of *Mycobacterium tuberculosis* H37Rv were grown using *mycobacterial* growth indicator tubes (MGIT). The inocula were prepared from Lowenstein–Jensen slants. To prepare an inoculum that was less than 15 days old from a culture grown on Lowenstein-Jensen medium, a suspension was prepared in saline and adjusted to a 1.0 McFarland standard. The suspension was vortexed for several minutes and was allowed to stand for 20 min for the initial settling of larger particles. The supernatant was transferred to an empty sterile tube and was allowed to stand for an additional 15 min. After being transferred to a new sterile tube, it was then adjusted to a 0.5 McFarland turbidity standard by visual comparison. A 1:5 dilution of the bacterial suspension was prepared, and 0.5 ml was inoculated into MGIT 7H12® (MGIT 960 system, Becton Dickinson, Sparks, USA) tubes containing test and control compounds[46].

The growth of the organism was monitored through fluorescent changes due to oxygen consumption in the medium during active growth. Aliquots (100 μl) of each herbal medicine was added to the MGIT tubes containing bacteria in Middlebrook 7H12® media, with the final DMSO concentration not exceeding 1.2%. The tubes were incubated at 37°C in MGIT system, and growth units (GU) were monitored for six days. All the remedies were tested at concentrations of 50 and 25 ug/ml[46].

For MIC99 evaluations, a 1% bacterial control culture was prepared in a drug-free MGIT tube and the MIC_{99} of the compound determined relative to the growth units of the control (GU–400). The MIC was determined as the lowest drug concentration that equals or lower than GU of the 1% bacterial culture. Controls that were also included are bacterial culture with DMSO (1.2%), isoniazid (INH) and bacterial culture only. All the herbal preparations were tested at two-fold decreasing concentration[46].

Results

In the present study, the susceptibility and minimum inhibitory concentration (MIC) of nine polyherbal medicines were determined against *M. tuberculosis* H37Rv, in vitro. The susceptibility testing revealed that all the remedies have anti-tubercular activity against *M. tuberculosis* H37Rv at concentrations below 50 μg/ml. Seven of these polyherbal preparations, namely; KWTa, KWTb, KWTc, HBfs, HBts, AL and FB showed activity at concentrations below 25 μg/ml, with the remaining remedies showing activity at concentrations between 25 and 50 μg/ml (Table 2).

All the remedies exhibited inhibitory activity against *M. tuberculosis* H37Rv with KWTa, HBfs and HBts as the most active remedies at 1.562 μg/ml, followed by AL remedy which showed growth inhibition at 3.125 μg/ml. The remaining preparations from KWTb, KWTc, HBss, EL and FB showed growth inhibition against *M. tuberculosis* at 25 μg/ml. However, isoniazid showed more inhibitory activity against *M. tuberculosis* H37Rv at 0.05 μg/ml when compared to the polyherbal remedies (Table 2).

Table 2. Susceptibility testing and minimum inhibition concentration (MIC_{99}) of nine polyherbal remedies against *M. tuberculosis* H37Rv using MGIT BACTEC 960 system

Polyherbal remedies	Susceptibility activity (µg/ml)	MIC_{99} of the remedies (µg/ml)
KWTa	< 25	< 1.562
KWTb	< 25	25
KWTc	< 25	25
HBfs	< 25	< 1.562
HBss	> 25	25
HBts	< 25	< 1.562
AL	< 25	3.125
EL	> 25	25
FB	< 25	25
Isoniazid (INH)	-	0.05

Discussion

Tuberculosis has been a major health problem for developing countries including South Africa. The increasing resistance of the disease to first and second line drugs has demanded the need for a new search for anti-*mycobacterial* agents that could be effective, efficient, non-toxic and cost effective[47].

The herbal preparations from KWTa, HBfs, HBts and AL showed a greater anti-*mycobacterial* activity, resulting in lower susceptibility patterns and MIC values observed. From observation, the aforementioned remedies contain a mixture of two or more of the following herbs: *Allium sativum, Strychnos decussata, Daucus carota, Hypoxis argentea, Rapanea melanophloeo* together with other herbs. Species of these plants have been investigated and shown to contain anthraquinones, glycosides, saponins, tannins, terpenoids, aloin, saponins, steroids and flavonoids[48,49,50]. Other compounds include alkaloids, terpenes, resin, monoterpenoids, sesquiterpenoids and phenols which show activity against *Mycobacterium tuberculosis*[51,15,52]. *Allium sativum* is a plant that has been reported as an established remedy for the treatment of tuberculosis[53]. It possesses variety of biological properties such as anti-cancer, anti-microbial, antioxidant, immunomodulatory, anti-inflammatory, hypoglycaemic and anti-cardiovascular properties[54]. Several studies conducted on the in vitro activity of *Allium sativum* against *Mycobacterium tuberculosis* revealed that this plant possesses anti-tubercular properties[41,42,53,53]. The presence of sulphur compounds such as allicin, ajoene, allylmethyltrisulfide, diallyltrisulfide, diallyldisulphide has been associated with the anti-tubercular activity of this *Allium sativum*[55].

Information on the use of *Strychnos decussate* as an anti-tubercular agent has not been reported. This study is the first to report the use of this plant as a remedy for the treatment of TB. However, it has been reported to possess anti-fungal activity[56]. *Daucus carota* is a root vegetable. There are only a few reports on the anti-tubercular activity of this plant[57,58]. However, it has been reported to be used as an anti-bacterial[59], anti-fertility[60], anti-oxidant[61], ophthalmic and stimulant[62], anti-septic, diuretic, hepatoprotective, anti-inflammatory[63,64], anti-helmintic, carminative[65], deobstruent, diuretic and galactogogue. According to the reports, phenolics, polyacetylenes, carotenoids, ascorbic acid and tocopherol are the most abundant phytonutrients present in this plant[66]. *Hypoxis argentea* has also been reported to be used as a remedy for the treatment of TB[58]. Species of the genus *Hypoxis* have been used as anti-bacterial, anti-fungal, anti-viral, anti-oxidant, anti-inflammatory, anti-diabetic, cardiovascular, anti-convulsant and anti-cancer[67,68,69,70,71]. The presence of several compounds, especially glucosides, sterols and sterolins could be responsible for the different activities found in *Hypoxis*[72]. *Rapanea melanophloeo* has been screened for activity and found active against drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis*[73,74]. This plant has been reported to contain bioactive compounds such as benzoquinones, saponins and tannins which could probably contribute to its activity[73].

The high activity of these polyherbal remedies against *M. tuberculosis* could be attributed to the presence of multiple active constituents which may act in synergy and produce greater anti-*mycobacterial* activity. This is an indication that many natural products are potential source of antimycobacterial agents[42].

Conclusion

This study has revealed that polyherbal remedies have the potential to cure tuberculosis. This is the first research work on the anti-*tuberculosis* activity of polyherbal medicines used for the treatment of tuberculosis in South Africa. The remedies might be potential sources of new anti-mycobacterial agents as they all showed activity against *M. tuberculosis*. However, the activity of these remedies and their active principles still require in vivo study in order to validate their potential as anti-*tuberculosis* agents.

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Conflict of interest

The authors declare no conflict of interest.

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CHAPTER SIX

The effect of polyherbal medicines used for the treatment of tuberculosis on other opportunistic organisms of humans infected with tuberculosis

CHAPTER SIX

TABLE OF CONTENT

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ORIGINAL ARTICLE

The Effect of Polyherbal Medicines Used for the Treatment of Tuberculosis on Other Opportunistic Organisms of Humans Infected with Tuberculosis

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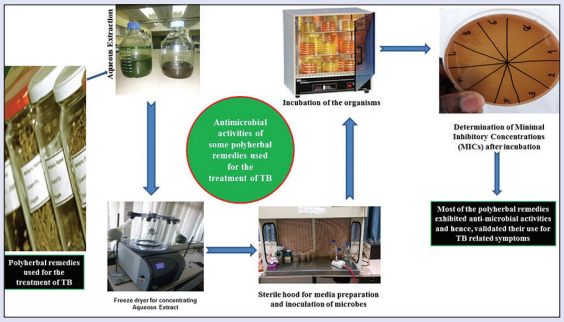
ABSTRACT

Background: In many immunocompromised patients, opportunistic bacterial and fungal infections are common. Polyherbal medicines examined in this study are used by the indigenous people of South Africa for the treatment of tuberculosis (TB) and other opportunistic infections associated with TB. **Objective:** To evaluate the antibacterial and antifungal activity of nine polyherbal remedies against four Gram-positive and Gram-negative bacteria respectively and three fungi. **Materials and Methods:** Agar dilution method was used to determine the minimum inhibitory concentration (MIC) of the remedies against the organisms. **Results:** The inhibitory activity of the polyherbal medicines based on the overall MIC revealed that HBfs and FB remedies were the most active remedies against the bacterial isolates at the concentration of 2.5 mg/mL, followed by HBts remedy at 5.0 mg/mL. However, the MIC valves of KWTa, KWTb, KWTc, HBss, EL and AL remedies were higher than 5.0 mg/mL which was the highest concentration used. Only KWTa remedy showed activity against *Aspergillus niger* and *Aspergillus fumigatus* with the MIC value of 2.5 mg/mL. While KWTc and HBts had the highest activity at 1.25 mg/mL against *Candida albicans*, the remaining remedies were active at 2.5 mg/mL. **Conclusion:** This study revealed that some of these polyherbal formulations have activities against some of the opportunistic bacterial and fungal isolates associated with TB patients. The capability of these remedies to inhibit the organisms is an indication that they are a potential broad-spectrum antimicrobial agent. However, the remedies that are inactive might contain stimulant effects on the immune system.

Key words: Antibacterial, antifungal, polyherbal medicines, tuberculosis

SUMMARY

- In the Eastern Cape Province of South Africa, no study has been reported on the effect of polyherbal remedies used for the treatment of TB on the opportunistic pathogen. This study therefore revealed that some of the polyherbal medicines possess activity against bacterial and fungal pathogens.



Abbreviations used: TB: Tuberculosis; MIC: Minimum Inhibitory Concentration; CFU/ML: Colony Forming Unit Per Mill.

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INTRODUCTION

The use of herbal medicines for the prevention and cure of various diseases has increased tremendously all over the world in the recent years. They are believed to be safe, effective, accessible, and free from serious adverse reactions.[1,2] These medicines are often "polyherbal" preparations made from the mixtures of various medicinal plants. Hence, they contain multiple bioactive constituents that interact with each other in the formulation and achieve extra therapeutic effectiveness.[3] Due to the wide therapeutic range and high efficiency of polyherbal formulations, they are used for the treatment of a vast number of diseases such as diabetics, arthritis, liver and kidney disorders, cough, asthma, fever, respiratory disorders, and tuberculosis (TB).[4-9]

TB is a chronic infectious disease and has remained one of the major public health problems in South Africa. The country ranks the third highest in the world with a high incidence of TB with about 80% of the population infected with latent TB.[10] The chronic nature of tubercular infection coupled with long-term administration of antibiotics not only leave the patients with impaired immunity but also predispose them to opportunistic pathogens.[11,12] The suppression of human defense mechanism during the course of active TB makes the patients vulnerable to pathogenic and opportunistic pathogens, and, as such, acquires fungal infection in addition to bacterial, viral and parasitic infections.[13,14] These organisms invade any part of the body and cause secondary infections.[15] In addition, with the increasing incidence of resistant strains of bacteria and fungi with commonly used anti-infective agents and the persistence of these

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organisms in immunocompromised patients, it is of great importance to find effective antimicrobial agents against the infections caused by these organisms.

The most common opportunistic bacterial infections in immunocompromised TB patients are members of the family Enterobacteriaceae, *Pseudomonas* spp. and *Staphylococcus* spp.[14] These bacteria have been extensively studied and found to cause infections in patients undergoing prolonged use of antibiotics and immunosuppressed individual.[14,16,17] The synergistic growth promoting association of fungi and *Mycobacterium* has been reported in pulmonary TB patients.[18] This is due to the prolonged use of chemotherapy with or without corticosteroids which promotes the growth and reproduction of opportunistic fungi and, in turn, aggravates the course of the underlying process in the lung tissues.[19] The major opportunistic fungal pathogens that have been reported to cause infections in TB patients include *Aspergillus* spp. causing aspergillosis, *Candida* spp. causing candidiasis, *Cryptococcus neoformans* causing cryptococcosis, and *Mucor* spp. causing mucormycosis.[12,18,20] Several researchers have investigated the antibacterial and antifungal activity of medicinal plants used for the treatment of TB in the province.[21,22] However, no study has been reported on the antimicrobial effect of polyherbal remedies used for the treatment of TB. Since opportunistic bacterial and fungal pathogens tend to cause diseases in TB patients, the study was, therefore, aimed at evaluating the antimicrobial effect of polyherbal medicines used for the treatment of TB in the Eastern Cape Province of South Africa, in other to determine whether they could also serve as antibacterial and antifungal agents.

MATERIALS AND METHODS

Sample collection

Polyherbal medicines evaluated in this study were purchased from herbal healers in five communities, namely, Alice (AL), East London (EL), Fort Beaufort (FB), Hogsback, and King Williams Town. These are some of the towns within the Amathole District Municipality of Eastern Cape Province, South Africa. Each of the aqueous formulation was already prepared and packaged into a clean 2-L container by the herbal healers. The remedies were called TB healing remedy. Thus, they were code-named according to their respective place of collection as follows; King Williams Town site A (KWTa), King Williams Town site B (KWTb), King Williams Town site C (KWTc), Hogsback first site (HBfs), Hogsback second site (HBss), Hogsback third site (HBts), EL, AL, and FB. The samples were transported to the laboratory for analysis.

Sample preparation

All the polyherbal preparations were filtered using Buchner funnel and Whatman No. 1 filter paper. The filtrate obtained was frozen at − 40°C and freeze-dried for 48 h using a freeze-dryer (VirTis BenchTop K, VirTis Co., Gardiner, NY, USA). Each of the samples was re-suspended in distilled water to yield 100 mg/mL stock solution.[23]

Microorganisms and media

The bacteria and fungi used in this study were chosen primarily on the basis of their importance as opportunistic pathogens of humans infected with TB.[12,22,24] Strains from the American Type Culture Collection (ATCC) were used for both assays. The bacteria used include four Gram-positive and Gram-negative bacteria, namely, *Staphylococcus aureus* ATCC 29213, *Bacillus cereus* ATCC 10702, *Enterococcus faecalis* ATCC 29212, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 100031, *Escherichia coli* ATCC 8739, and *Salmonella typhimurium* ATCC 13311.

For the antifungal analysis, the fungal strains used include *Candida albicans* ATCC 10231, *Aspergillus fumigatus* ATCC 204305, and *Aspergillus niger* ATCC 16888. Mueller-Hinton Agar (MHA), Mueller Hinton Broth (MHB), Sabouraud Dextrose Broth (SDB), and Sabouraud Dextrose Agar (SDA) used were obtained from BioLab and were prepared according to the manufacturer's instructions.

Preparation of inocula

For the bacterial inoculum preparation, all the test bacteria strains that were maintained on nutrient agar slants were recovered in sterile MHB and incubated overnight at 37°C. In other to obtain distinct colonies, the 24 h old cultures were diluted 1:100 v/v in fresh sterile MHB and cultured on MHA overnight at 37°C. The colony suspension method of EUCAST[25] was used for the preparation of the inoculum. Identical colonies from the culture were suspended in 0.85% sterile saline, adjusted with saline and compared with 0.5 McFarland standards to obtain a suspension density equivalent to 10^6 CFU/mL. The suspensions were confirmed by spectrophotometric reading at 600 nm. The cell suspensions were finally diluted 1:100 by transferring 0.1 mL of the bacterial suspension into 9.9 mL of sterile broth to give an approximate inoculum of 10^4 CFU/spot.[26] The inocula suspensions were used for inoculation within 15 min.

For the fungal inoculum preparation, modified method of Therese *et al.*[27] was used for the analysis. The fungi strains were freshly sub-cultured on SDA and incubated at 25°C for 72 h. About 1 cm^2 of 3-day-old spore producing cultures were dropped in sterile distilled water and vortexed for 30 s to release the fungal spores. The spore density of each fungus was adjusted with a spectrophotometer at 580 nm to obtain a final concentration of approximately 10^5 spores/mL. Cell suspensions were finally diluted to 10^4 CFU/spot.[28] For the *Candida* spp., the inocula were prepared by adding 1.0 mL of overnight *Candida* cultures to 9.0 mL of SDB to yield 10^4 CFU/spot of the inoculum.[29]

Antibacterial and antifungal minimum inhibitory concentration assays

Determination of the minimum inhibitory concentration of the herbal remedies against bacterial isolates

The antibacterial activity of the polyherbal remedies was carried out using the agar dilution method of Afolayan and Meyer[30] with slight modification. MHA was prepared according to the manufacturer's instructions and placed in a water bath at 50°C to prevent solidification. From the stock solution of each remedy (100 mg/mL), different concentrations of 5.0, 2.5, 1.25, 0.625, 0.3125, and 0.15625 mg/mL were prepared and incorporated in the molten agar (three replicates). The agar containing the remedy was poured into sterile Petri dishes, swirled carefully and allowed to cool. The controls used were blank plates containing only MHA, another plates containing MHA and 1.0 mL of distilled water which is the solvent of extraction (negative control), and plates containing 0.0125–0.000391 mg/mL of ciprofloxacin serves as the positive controls. Ten microliters of the standardized bacterial cultures were streaked in a radial pattern on the solidified agar remedy plates. The plates were incubated at 37°C for 24–72 h. The concentration at which there was no visible growth of the organism on the agar plates was considered the minimum inhibitory concentration (MIC) of the remedy.[25]

Determination of the minimum inhibitory concentration of the herbal remedies against fungal isolates

The antifungal activity of the remedies was evaluated using the agar dilution method of Therese *et al.*[27] with slight modification. SDA was prepared according to the manufacturer's instructions and placed

in a water bath at 50°C to prevent solidification. Before congealing, different volumes of the herbal remedies were added to the SDA to have concentrations ranging between 0.15625 and 5.0 mg/mL. The controls used were blank plates containing only SDA, another containing SDA and 1.0 mL of distilled water (negative control), and plates containing 12.5–0.391 μg/mL of amphotericin-B (positive controls). Ten microliters of the final suspensions were placed on the solidified agar remedy plates. The plates were incubated at 28°C for 48–96 h. The concentration at which there was no visible growth of the organism on the agar plates was considered the MIC of the remedy.[27]

RESULTS

Effect of the herbal remedies on bacterial isolates

Evaluation of the antibacterial activity of the nine polyherbal remedies against eight bacterial isolates revealed that three of the herbal formulations, namely, HBfs, FB, and HBts exhibited antibacterial properties [Table 1]. The growth of *K. pneumoniae*, *P. aeruginosa*, *E. coli*, *E. faecalis*, *B. cereus,* and *S. aureus* was inhibited by HBfs remedy at a concentration of 2.5 mg/mL while *Salmonella typhimurium* and *Streptococcus pyogenes* were inhibited at the MIC of 5.0 mg/mL. Furthermore, FB preparation was active against six bacteria, namely, *P. aeruginosa, S. typhimurium, E. faecalis, S. pyogenes*, *B. cereus,* and *S. aureus* at the MIC of 2.5 mg/mL, while *K. pneumoniae* and *E. coli* were inhibited at 5.0 mg/mL. HBts remedy showed activity at 5.0 mg/mL against eight isolates except on *B. cereus* which was resistant to the remedy. The MIC of KWTa, KWTb, KWTc, HBss, EL, and AL remedies against the bacterial isolates was higher than 5 mg/mL which was the highest concentration used. However, the standard antibiotic (ciprofloxacin) used as a positive control inhibited the isolates at the concentration of 0.0015625 mg/mL except on *S. typhimurium* were the MIC at 0.003125 mg/mL [Table 1]. The inhibitory activity of the herbal formulations based on the overall MIC revealed that HBfs and FB remedies were the most active against the bacterial isolates followed by HBts, while the remaining formulations showed no activity at the concentration used [Table 1].

Effect of the herbal remedies on fungal isolates

The effects of the remedies against the fungal isolates are shown in Table 2. Among the nine herbal formulations tested, only KWTa remedy was active against *A. niger* and *A. fumigatus* with the MIC value of 2.5 mg/mL [Table 2]. Other remedies; KWTb, KWTc, HBfs, HBss, HBts, AL, EL, and FB were inactive against *Aspergillus* spp. at the concentration used in this study. However, all the remedies showed activity against *C. albicans*. The highest activity was recorded in KWTc and HBtt remedies with the MIC value of 1.25 mg/mL while the remaining remedies had the MIC of 2.5 mg/mL. The activity of these remedies against the yeast (*C. albicans*) alone suggest that the remedies might not have high efficacy with a broad spectrum of antifungal activity compared to the drug (amphotericin B) used as a positive control which inhibited the isolates at the concentration of 0.391 μg/mL [Table 2].

DISCUSSION

Polyherbal formulations are well known used Ayurvedic medicines for their effectiveness against a wide range of diseases.[31] They contain multiple bioactive constituents that act synergistically and achieve extra therapeutic effectiveness. Polyherbal formulations have been reported to possess activity against opportunistic antibacterial and antifungal isolates.[32-36] However, herbal formulations examined in this study are used by TB-patients for the treatment and management of TB, especially, in the Eastern Cape Province, South Africa.

Table 1: Minimum inhibitory concentration of polyherbal remedies used for the treatment of tuberculosis on bacterial isolates associated with tuberculosis infection

Bacteria species	Gram-positive/ negative	KWTa (mg/mL)	KWTb (mg/mL)	KWTc (mg/mL)	HBfs (mg/mL)	HBss (mg/mL)	HBts (mg/mL)	AL (mg/mL)	EL (mg/mL)	FB (mg/mL)	Ciprofloxacin (mg/mL)
Klebsiella pneumoniae ATCC 100031	Negative	>5	>5	>5	2.5[a]	>5[c]	5	>5	>5	5	0.0015625
Pseudomonas aeruginosa ATCC 27853	Negative	>5	>5	>5	2.5	>5	5	>5	>5	2.5	0.0015625
Salmonella typhimurium ATCC 1331	Negative	>5	>5	>5	5[b]	>5	5	>5	>5	2.5	0.0031250
Escherichia coli ATCC 8739	Negative	>5	>5	>5	2.5	>5	5	>5	>5	5	0.0015625
Enterococcus faecalis ATCC 29212	Positive	>5	>5	>5	2.5	>5	5	>5	>5	2.5	0.0015625
Streptococcus pyogenes	Positive	>5	>5	>5	5	>5	5	>5	>5	2.5	0.0015625
Bacillus cereus ATCC 10702	Positive	>5	>5	>5	2.5	>5	5	>5	>5	2.5	0.0015625
Staphylococcus aureus ATCC 29213	Positive	>5	>5	>5	2.5	>5	>5	>5	>5	2.5	0.0015625

[a]MIC; [b]Maximum concentration of the polyherbal medicines tested; [c]Not active. KWTa: King Williams Town site A; KWTb: King Williams Town site B; KWTc: King Williams Town site C; HBfs: Hogsback first site; HBss: Hogsback second site; HBts: Hogsback third site; AL: Alice; EL: East London; FB: Fort Beaufort; ATCC: America Type Culture Collection; MIC: Minimum inhibitory concentration

Table 2: Minimum inhibitory concentration of polyherbal remedies used for the treatment of tuberculosis on fungal isolates associated with tuberculosis infection

Fungi species	KWTa (mg/mL)	KWTb (mg/mL)	KWTc (mg/mL)	HBfs (mg/mL)	HBss (mg/mL)	HBts (mg/mL)	AL (mg/mL)	EL (mg/mL)	FB (mg/mL)	Amphotericin-B (µg/mL)
Aspergillus niger ATCC 16888	2.5	>5	>5	>5	>5[c]	>5	>5	>5	>5	0.78
Aspergillus fumigatus ATCC 204305	2.5	>5	>5	>5	>5	>5	>5	>5	>5	0.78
Candida albicans ATCC 10231	2.5	2.5	1.25[a]	2.5[b]	2.5	1.25	2.5	2.5	2.5	0.39

[a]MIC; [b]Maximum inhibitory concentration; [c]Not active. KWTa: King Williams Town site A; KWTb: King Williams Town site B; KWTc: King Williams Town site C; HBfs: Hogsback first site; HBss: Hogsback second site; HBts: Hogsback third site; AL: Alice; EL: East London; FB: Fort Beaufort; ATCC: American Type Culture Collection; MIC: Minimum inhibitory concentration

In this study, three of the herbal formulations exhibited antibacterial properties against both Gram-negative (*P. aeruginosa*, *K. pneumoniae*, *E. coli,* and *S. typhimurium*) and Gram-positive bacteria (*E. faecalis*, *S. pyogenes, B. cereus,* and *S. aureus*). This is an indication that the herbal preparations possess good antibacterial activity against the opportunistic bacteria capable of causing infection in TB-patients when compared with other remedies. From the results, the response of the bacteria to the remedies varied among the isolates and the concentrations. The variation in the MIC of the polyherbal remedies could be due to the mode of action, the differences in cell wall composition and/or genetic content of the organisms[37] as well as the synergistic effects of different phytochemicals present in the herbal formulations. The antibacterial activity of HBfs, FB, and HBts remedies at relatively minimal concentrations could be attributed to the bioactive constituents present in the remedies at different concentrations, its ability to damage the cell walls to allow the active compounds to absorb, diffuse, penetrate and interact with the target sites and potent enough to inhibit, or kill microbial agents.[38]

Furthermore, the results showed that all the remedies were active against the growth of only the yeast (*C. albicans*). The resistance of *Aspergillus* spp. suggests that the remedies might not have high efficacy with a broad spectrum of antifungal activity when compared with amphotericin-B which showed activity against the three fungi. However, the activity of KWTa remedy could be attributed to the presence of bioactive antifungal agents which are not in the other remedies. In general, the poor growth inhibitory activity against some of the organisms demonstrated by some of these remedies could be attributed to the solvent (water) used by the herbal healers for their preparation. Aqueous extracts have been reported to be generally less active as described in many previous studies.[37,39,40] The activity of these remedies on the opportunistic pathogens does not mean that they are inactive. They may act by stimulating the immune system of the patient, or by creating internal conditions that are unfavorable for the multiplication of the microorganism.[21]

CONCLUSION

This study revealed that some of these polyherbal formulations have activities against some of the opportunistic bacterial and fungal isolates associated with TB patients. The capability of these remedies to inhibit the organisms is an indication that they are a potential broad-spectrum antimicrobial agent. However, the remedies that are inactive might contain stimulant effects on the immune system.

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Conflicts of interest

There are no conflicts of interest.

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CHAPTER SEVEN

Toxicological Evaluation of Polyherbal Medicines used for the Treatment of Tuberculosis in Eastern Cape, South Africa

CHAPTER SEVEN

TABLE OF CONTENT

Chapters **Page No**

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Research Article
Toxicological Evaluation of Polyherbal Medicines used for the Treatment of Tuberculosis in Eastern Cape, South Africa

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Abstract

Background: Polyherbal remedies are widely used for the treatment and management of various diseases in developing countries. These remedies often contain active pharmacological compounds, thus, the evaluation of herbal remedies used for the treatment of tuberculosis in the Eastern Cape province for their toxicity is of great importance. **Materials and Methods:** Nine polyherbal medicines used for the treatment of tuberculosis were assayed for their toxicity using hatchability success and larval mortality of brine shrimp (*Artemia salina* Leach). These remedies were liquid preparations and coded according to their respective place of collection, viz., King Williams Town site A, King Williams Town site B, King Williams Town site C, Hogsback first site, Hogsback second site, Hogsback third site, East London, Alice and Fort Beaufort. **Results:** The percentage hatchability success of 44.42, 42.96 and 39.70% were observed in cysts incubated with herbal preparations from King Williams Town site A, Hogsback first site and Hogsback third site, respectively. The hatching success in these remedies was significantly higher than the positive control (nystatin) and the negative control (sea water) at $p<0.05$. The herbal preparations from King Williams Town site A and East London exhibited significantly more inhibitory hatchability effects with minimum inhibitory concentration values of 2.4 and 2.8 mg mL^{-1}, respectively. The mortality of *A. salina* nauplii incubated in Alice, King Williams Town site B and King Williams Town site C remedies was significantly higher than when larvae was incubated in both controls. Based on Meyer's index, the LD_{50} of each polyherbal medicine was between 2.9-4.0 mg mL^{-1}, the LD_{50} values greater than 1 mg mL^{-1}. **Conclusion:** The polyherbal remedies evaluated in this study are considered non-toxic and are therefore safe for the patients. However, further *in vivo* toxicity tests are required to validate the safe use of these polyherbal remedies.

Key words: Polyherbal, *Artemia salina*, hatchability assay, lethality test, tuberculosis

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INTRODUCTION

Polyherbal medicines are extensively used in many developing countries for the prevention and treatment of various diseases such as diabetics, wound infection and tuberculosis. These preparations are freely hawked in South Africa and are prevalent in other African societies. They are readily available and affordable. Polyherbal preparations contain a mixture of three or more medicinal plants. The rationale behind the combination of these herbs could not be justified by the traditional healers as it was a practise that they found to be effective. A review of studies into medicinal plants used to treat various diseases revealed that 80% of the people in South Africa make use of herbal remedies for the treatment of ailments at some stage in their life[1].

Despite the widespread use of polyherbal remedies for the treatment of several illnesses, little is known about their toxicity and safety. The evaluation of the toxic action of these remedies is important in order to acquire their maximum benefits to humans, even though they have been proven to be efficacious in pharmacological studies or by clinical evaluation[2]. The reports of patients experiencing adverse effects such as diarrhoea, abdominal pain, ulcer, dizziness, loss of appetite, abortion of pregnancy and stroke caused by these remedies are on the rise[3]. Other dangerous effects include heart attacks, heart-rate irregularities, liver toxicity, seizures, psychoses and death[4,5]. Hence, the need to analyse the physiological effects of polyherbal remedies is imperative.

This toxicity test aims at establishing the therapeutic index, LD_{50}. The greater the index the safer the remedies, the smaller this margin the more chances of producing unwanted effects[6]. In the literature, there is no information on the toxicological study of polyherbal medicines in South Africa to authenticate their usage and guarantee the safety of the users. Thus, the objective of the current study was designed to investigate the toxicity of polyherbal medicines used for the treatment of tuberculosis in the Eastern Cape province using brine shrimp assay.

MATERIALS AND METHODS

Collection of polyherbal medicines: Polyherbal medicines evaluated in this study were purchased from hawkers and healers in five communities namely, Alice, Fort Beaufort, Hogsback, King Williams Town and East London, all within the Amathole district municipality of the Eastern Cape province, South Africa. Each of the liquid remedy was already prepared by the seller and packaged in a 2 L container. They were transported to Medicinal Plants and Economic Development Research Centre, University of Fort Hare for analysis. Each medicine was labelled and coded according to their respective place of collection; namely: King Williams Town site A (KWTa), King Williams Town site B (KWTb), King Williams Town site C (KWTc), Hogsback first site (HBfs), Hogsback second site (HBss), Hogsback third site (HBts), East London (EL), Alice (AL) and Fort Beaufort (FB).

Sample preparation: Each remedy was filtered using Buchner funnel and Whatman No. 1 filter paper. The filtrate obtained was frozen at -40°C and freeze dried for 48 h using a freeze dryer (Vir Tis benchtop K, Vir Tis Co., Gardiner, NY). The resulting sample was packaged in a clean container. For brine shrimps experiment, each sample was re-suspended in sea water to yield a 20 mg mL^{-1} stock solution.

Assay procedure: The assay was carried out using modified method of Otang *et al.*[7]. Five petri dishes containing 30 mL of filtered sea water each was prepared and a two-fold dilution was set up to yield a series of concentrations (1, 0.5, 0.25, 0.125 and 0.0625 mg mL^{-1}) of the polyherbal medicines. A positive control was prepared in test tubes containing nystatin in seawater (30 µL mL^{-1}), while petri dishes containing sea water only served as the blank control.

Brine shrimp (*Artemia salina*) hatchability assay: The *Artemia salina* cysts (Sera, Heidelberg, Germany) were obtained from an aquaculture shop in East London, South Africa. These were used to evaluate the hatchability success of *A. salina* cysts against different concentrations of the polyherbal medicines[8]. Briefly, different concentrations (0.0625-1 mg mL^{-1}) of the herbal remedies and positive control were prepared in sea water. The *A. salina* cysts were stocked at a density of 15 individuals per petri dish containing 30 mL of the incubation medium at varying concentrations. The plates were partly covered and incubated at 28°C under constant illumination in a digital incubator (MRC Laboratory equipment, model LE-509) and aeration. Thereafter, the petri dishes were examined with the aid of a hand lens against a white background that allowed the nauplii to be separated from the shells. The number of free nauplii in each petri dish was counted after every 12 h for 72 h. The percentage of hatchability success was calculated by comparing the number of hatched nauplii in a chosen concentration with the total number of cysts stocked[9]. The minimum concentration of the polyherbal medicines (or control drug) that inhibited the hatching of the cysts was taken as the MIC. All the assays were carried out in three replicates.

Brine shrimp lethality assay: This was carried out in order to determine the toxic level of the polyherbal preparations. About 1 g of the shrimp cysts were introduced into 1 L sea water. The beaker was partly covered and incubated at 28°C under constant illumination in a digital incubator and aeration. After 36 h of hatching, the phototropic nauplii were collected and an aliquot (0.15 mL) containing 15 nauplii was pipette into each petri dish for each remedy solution and controls. The numbers of surviving larvae in each petri dish were counted after every 12 h. The setup was allowed to remain for 72 h under constant illumination. Finally, the percentage of deaths was calculated. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation[9].

The percentage of mortality was calculated as follows:

$$\text{Mortality (\%)} = \frac{\text{(Total nauplii-Alive nauplii)}}{\text{Total nauplii}} \times 100\%$$

Determination of MIC 50 and LD_{50}: The percentage hatchability success and mortality data obtained from the five concentrations of each polyherbal remedy was used to construct a dose-response curve. The Minimum Inhibitory Concentration (MIC 50) was determined as the concentration of the polyherbal remedy/control drug that inhibited hatching of 50% of the cysts. The LD_{50} was taken as the concentration required for producing 50% mortality[10]. The LD_{50} values were determined from the best-fit line obtained by linear regression analysis of the percentage lethality versus the concentration.

Statistical analysis: Statistical analysis was performed on MINITAB version 12 for windows (Minitab Inc., Pennsyvania, USA). One-way analysis of variance was used to test for the effect of concentration and time of exposure of the herbal remedies on the hatchability success of the cysts and mortality of the larvae in comparison to controls[11]. The study samples were tested for normality. The p-value less than 0.05 were considered significantly different.

RESULTS AND DISCUSSION

Brine shrimp hatchability assay: The percentage hatchability success of *Artemia salina* cysts incubated with different polyherbal medicines are shown in Fig. 7.1. High hatchability successes of 44.42, 42.96 and 39.70% were observed in the cysts incubated with polyherbal remedies from KWTa, HBfs and HBts, respectively and were significantly higher than the controls. On the other hand, the hatching success of brine shrimps in KWTc, HBss, FB and EL remedies were higher than those incubated in nystatin but lower than those in the sea water. The herbal preparations from AL and KWTb exhibited more inhibitory effects with hatchability successes of 26.59 and 20.37%, respectively, which were lower than the controls. The inhibitory effects of the remedies on hatchability were expressed as MIC values (Table 7.1). The remedies from KWTa and EL exhibited more inhibitory hatchability effects with MIC values of 2.4 and 2.8 mg mL^{-1}, respectively. However, KWTb, FB and HBts preparations had the highest value of 3.9, 3.8 and 3.9 mg mL^{-1}, respectively, indicating that they could be less toxic among the 9 remedies (Table 7.1).

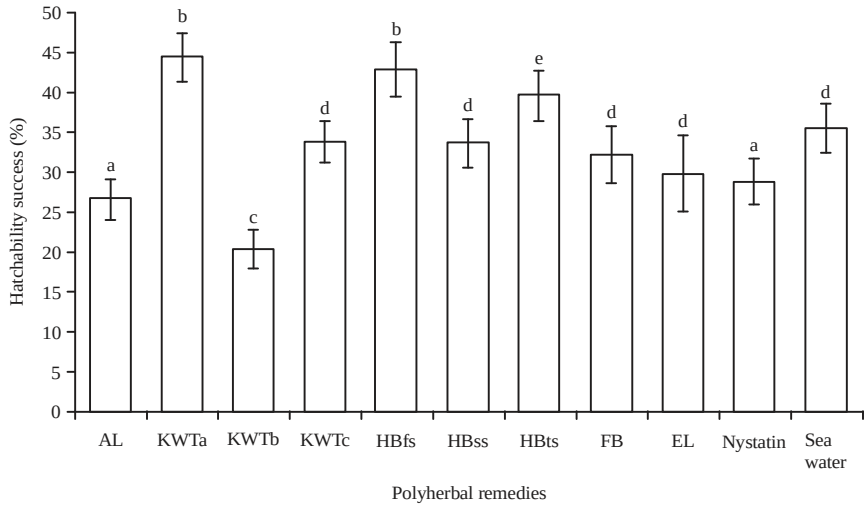

Fig. 7.1: Percentage hatchability success of *A. salina* cysts incubated in different polyherbal medicines. Means are values of five concentrations for each remedy ±SD. Bars with different letters are significantly different ($p<0.05$)

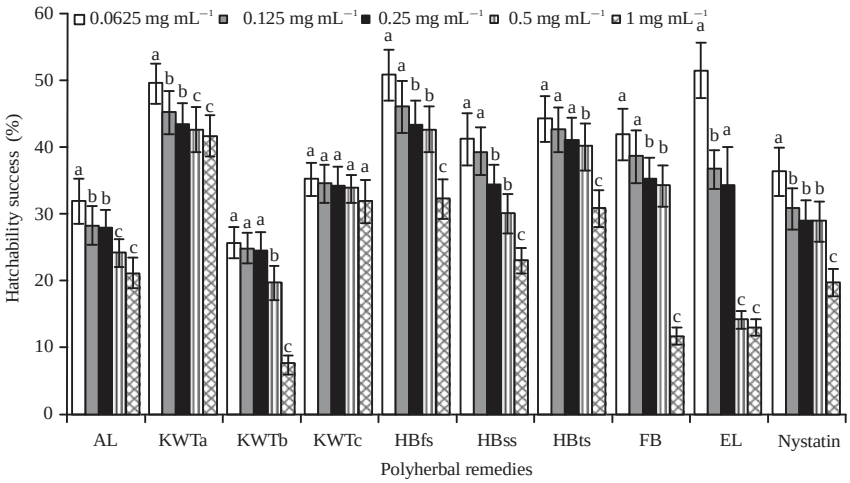

Fig. 7.2: Percentage hatchability success of *A. salina* cysts incubated in different concentrations of polyherbal medicines. Bars with different letters are significantly different

Table 7.1: Hatchability and lethality of *A. salina* incubated in different concentrations of polyherbal medicines

	Hatchability assay		Lethality assay	
Remedies	MIC 50 (mg mL^{-1})	R^2 (%)	LD_{50} (mg mL^{-1})	R^2 (%)
AL	3.0±0.87^a	96	3.0±0.03^a	99
KWTa	2.4±0.97^b	88	3.1±0.00^b	97
KWTb	3.9±0.23^c	74	3.8±0.68^c	80
KWTc	3.5±0.99^d	89	2.9±0.64^d	91
HBfs	3.4±0.72^e	88	3.4±0.15^e	95
HBss	3.3±0.56^f	96	3.2±0.04^f	96
HBts	3.8±0.83^g	78	3.4±0.55^g	92
FB	3.9±0.15^h	74	3.7±0.00^b	84
EL	2.8±0.04^i	93	4.0±0.01^h	70
Nystatin	3.3±0.68^j	86	3.5±0.37^i	92

Data are Means±SD of three replicates. Means with different superscript in the same column are significantly different ($p<0.05$), R^2 (%) is the coefficient of determination of the regression equation

Effect of polyherbal medicines concentration on hatchability success: The hatchability success of *A. salina* cysts decreased significantly with increasing concentrations of the polyherbal medicines (Fig. 7.2). The herbal preparations from EL, HBfs, KWTa and HBts had the highest hatchability success in the lowest concentration. In AL and HBss remedies, the hatching success decreased with increase in concentrations of the treatments. However, the inhibition of the hatching success in FB preparation was significantly decreased at 1 mg mL^{-1}. With increasing concentration from 0.0625 mg mL^{-1}, the hatchability success of the cysts incubated with KWTb remedy elicited more inhibitory effect on the hatching success at 1 mg mL^{-1} (Fig. 7.2). Thus, the decrease observed in the hatchability success of *A. salina* cysts as the concentrations increased could be as a result of the relative concentration of toxic metabolites present in the remedies as the concentration increases. However, none of the preparations exhibited total inhibition at 1 mg mL^{-1}, this might be due to the cysts possessing a resistant cyst stage which makes it tolerant to wide range of salinities[12].

Effect of exposure time on hatchability success: The effect of exposure time on hatchability obtained in this study revealed that the sensitivity of *A. salina* to polyherbal therapies was strongly dependent on exposure period (Fig. 7.3). The lowest hatchability success of the cysts was observed at 12 h of exposure in all the remedies. Higher hatchability success in incubations of KWTa, KWTc, HBfs, HBss, HBts and EL remedies at 24 h exposure were significantly higher than the controls. At 36 h of exposure, the hatchability successes of 49.78, 54 and 51.11% was observed in KWTa, HBfs and HBts medicines, respectively. The hatching success of *A. salina* cysts into nauplii in incubations of AL, HBfs, HBss, HBts, FB and EL remedies at 48 h of exposure ranged from 39.56-59.56%. The optimal hatching of cysts to yield a large number of nauplii is achieved with 48 h of exposure[13]. In AL, KWTb and EL remedies, the hatchability success was lower when compared with both controls at 60 h of exposure. Likewise, the hatching success of all the remedies except KWTa was lower than in the sea water at 72 h of exposure. *Artemia* is highly vulnerable to toxins and chemical metabolites at the early developmental stages[14], this could have led to the very low hatchability success of the cysts observed at 12 h of exposure. Thus, the resistant cysts stage of *A. salina* to higher salinities makes the hatchability assay less desirable assay than the lethality test for the preliminary screening of herbal remedies' toxicity test. The use of freshly hatched nauplii has been used to circumvent the toxic tolerant stage of *A. salina* cysts and this increases the sensitivity of the lethality assay[11,7].

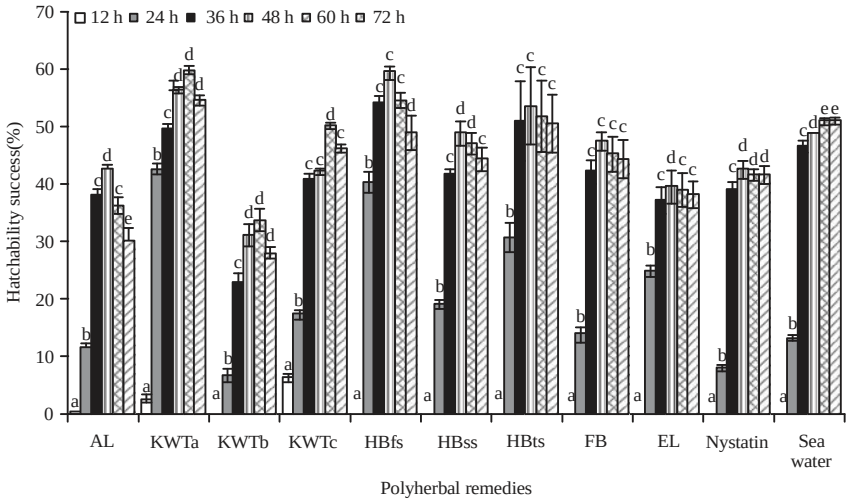

Fig. 7.3: Percentage hatchability success of *A. salina* cysts incubated at different durations in the polyherbal remedies. Bars with different letters are significantly different

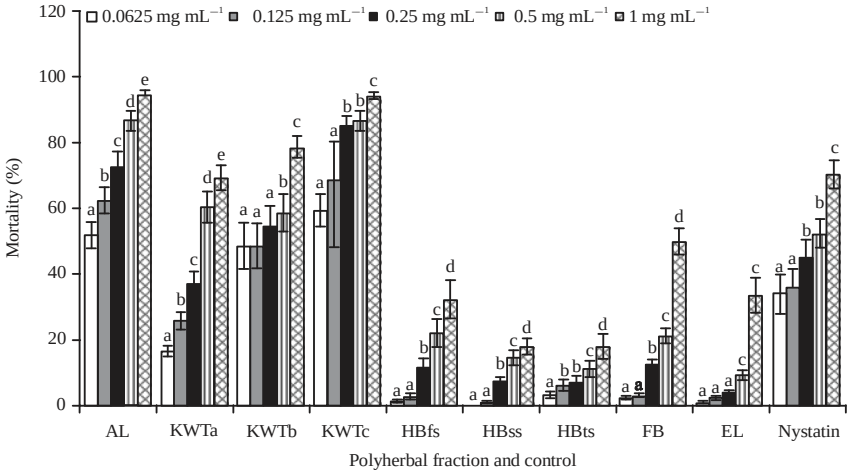

Fig. 7.4: Percentage mortality of *A. salina* nauplii incubated in different concentrations of the polyherbal medicines. Bars marked with different letters are significantly different

Effect of varying concentrations on brine shrimp mortality: The effect of varying concentrations of polyherbal remedies on the mortality of larvae is shown in Fig. 7.4. The degree of mortality of nauplii in all the remedies was in concentration dependent fashion. The percentage mortality of larvae in HBfs, HBss, HBts, EL and FB remedies was significantly lower than the positive control. Maximum mortalities of 94.63, 78.72 and 94.67% occurred at the highest concentrations of 1 mg mL^{-1} in incubations of AL, KWTb and KWTc, respectively, which was significantly higher than the positive control. It could be deduced that the polyherbal remedies have both toxicological and pharmacological activities based on the dosage administered.

Effect of exposure time on brine shrimp mortality: The effect of remedies on the larvae over a period of time was carried out to determine the sensitivity of the larvae to toxic secondary metabolites present in the remedies. The mortality of nauplii incubated in all the remedies increased exponentially with time (Fig. 7.5). The observed result was depended on the length of incubation period as earlier reported by Otang *et al*.[7]. Exposure of the larvae for lesser periods (<36 h) in HBfs, HBss, HBts and EL remedies did not induce mortality when compared with the controls. However, increase in mortality was observed in AL, KWTa, KWTb and KWTc remedies which was significantly higher than the controls, except at 72 h in nystatin. Brine shrimp nauplii attain the second and third instars of their life cycle within 48 h of exposure, thus revealed

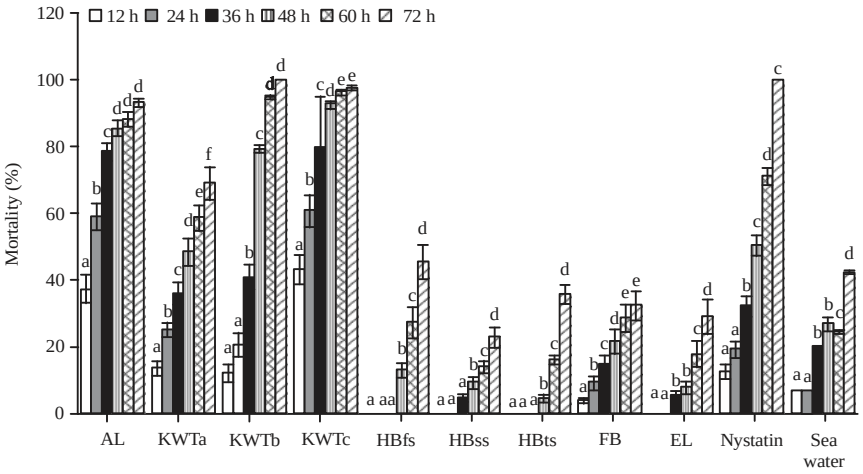

Fig. 7.5: Percentage mortality of *A. salina* nauplii incubated at different durations in the polyherbal remedies. Bars with different letters are significantly different

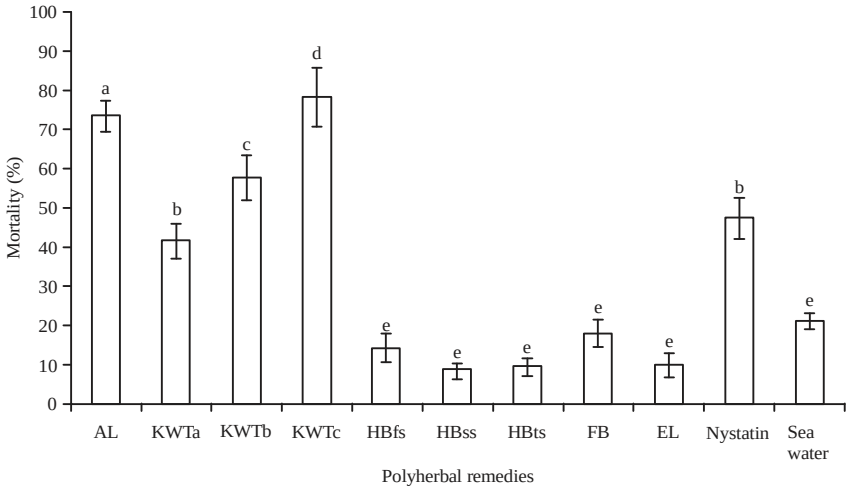

Fig. 7.6: Percentage mortality of *A. salina nauplii* incubated in different polyherbal medicines. Means are values of five concentrations for each polyherbal remedy±SD. Bars marked with different letters are significantly different

their greatest sensitivity to toxins at this time[15]. However, these findings revealed that the maximum sensitivity of the nauplii to the remedies was attained at 72 h of exposure. This could probably be due to the presence of nutritive metabolites in the remedies. Thus, the survival of high number brine shrimp nauplii in all the remedies after 60 h of exposure could be explained by the presence of non-toxic metabolites in the polyherbal medicines.

Brine shrimp lethality assay: Brine shrimp lethality results and LD_{50} values obtained are shown in Fig. 6 and Table 1, respectively. The larvae mortality of A. salina nauplii incubated in AL, KWTb and KWTc remedies was significantly higher than when larvae are incubated in both controls. However, lower larvae mortality was observed in the remedies from HBfs, HBss, HBts, FB and EL. Also, the number of larvae mortality was reduced in KWTa remedy than in nystatin but higher than those in sea water. While, EL remedy exhibited more lethality effects with LD value of 4.0, the lowest lethality effects was observed in KWTc remedy (Table 7.1).

In evaluating herbal preparations for toxicity, the LD_{50} values are commonly expressed either by comparison with Meyer's or to Clarkson's toxicity index. The extracts with LD_{50} less than 1000 µg mL^{-1} are considered as toxic, while extracts with LD_{50} greater than 1000 µg mL^{-1} are considered as non-toxic[13]. Clarkson classified cytoxicity as non-toxic when LD_{50} is above 1000 µg mL^{-1}, low toxic when the LD_{50} is between 500 and 1000 µg mL^{-1}, medium toxic when the LD_{50}

is between 100-500 µg mL^{-1}, while extracts with LD_{50} of 0-100 µg mL^{-1} are highly toxic[16]. According to these benchmarks, the LD_{50} of the nine polyherbal medicines varied between 3.0-4.0 mg mL^{-1}, these estimated LD_{50} values are greater than 1000 µg mL^{-1} (Fig. 7.6, Table 7.1). Thus, based on the criterion of toxicity, these remedies are considered non-toxic.

CONCLUSION

The findings from this study have shown that polyherbal remedies used for the treatment of tuberculosis in the study area exhibited non-toxic level i.e., LD_{50} values greater than 1 mg mL^{-1} with brine shrimp toxicity assays. Thus, they are considered safe for the patients. However, further *in vivo* toxicity test are required to validate the use of these polyherbal remedies.

ACKNOWLEDGMENT

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CHAPTER EIGHT

Evaluation of some important vitamins and mineral nutrients in polyherbal medicines used for the treatment of tuberculosis in the Eastern Cape Province, South Africa

This chapter has been accepted for publication in *International Journal of Phytomedicine*

CHAPTER EIGHT

TABLE OF CONTENT

Chapters **Page No**

Evaluation of some important vitamins and mineral nutrients in polyherbal medicines used for the treatment of tuberculosis in the Eastern Cape Province, South Africa

Abstract

The aim of this study was to determine the nutritive properties of nine polyherbal formulations, used for the treatment of tuberculosis, in order to validate their ability in boosting the immune system of tuberculosis patients. The remedies were analysed for their nutritive properties using an inductively coupled plasma optical emission spectrometer. The vitamins A, E and C were also determined using standardized methods. The polyherbal preparations were found to be rich in vitamins and mineral nutrients. Calcium was the highest mineral nutrient detected, while the lowest nutrient was phosphorus. Quantitatively, calcium and magnesium contents in the remedies ranged from 973.30 to 6503.30 mg/100g and 80.00 to 406.00 mg/100g respectively. The amount of phosphorus and potassium was between 20.00 and 263.30 mg/100g; 160.00 and 2050.00 mg/100g respectively. Micro minerals such as iron, manganese, zinc, aluminium and copper were also detected. Iron was the highest nutrient in the majority of the polyherbal preparations while the lowest value was recorded for copper. However, vitamin C was absent in the herbal preparations while vitamin A and E were detected. Thus, these polyherbal formulations contain the essential vitamins and nutrients that are probably boosting the immune system of tuberculosis patients thereby aiding their recovery.

Introduction

Tuberculosis (TB) is one of the deadliest diseases and has remained a major public health threat in most parts of the world (Chakraborty et al., 2014). Currently, it is estimated that over 2 billion people are infected with TB, with 8.6 million new cases per year; leading to the death of 1.3 million, including 320,000 deaths of HIV-TB co-infected individuals (WHO, 2013a; Baldwin et al., 2015). In South Africa, about 80% of the population is infected with latent TB among the age group 30-39 years (TBFACTS, 2015). The disease is related to poverty, under-nutrition and poor immune function (WHO, 2013b). TB and malnutrition constitute a substantial problem in the developing countries. Patients with active tuberculosis usually have low nutritional status, thus, suffer macronutrient and micronutrient malabsorption, reduction in appetite and altered metabolism of the body (Gupta et al., 2009).

Vitamins and minerals are essential needed for normal growth and maintenance of body functions. The minerals (macro and micronutrients) work together for tissue regeneration, cellular integrity and play important roles in the treatment of tuberculosis (Gupta et al., 2009; WHO, 2013b). However, vitamins are bio-molecules that maintain the physiology of the body and boost the immune system. They are responsible for a spectrum of vital functions in the body due to their anti-oxidant, pro-oxidant, anti-inflammatory effects and metabolic functions. While vitamin A, C and E largely contribute to the anti-oxidant system of the human body (Ciccone et al., 2013; Wahlqvist, 2013; Chakraborty et al., 2014), vitamins C and E are effective in improving the immune responses to tuberculosis when given as an adjuvant to multidrug tuberculosis therapy (Safarian et al., 1989).

The use of herbal medicines for the treatment of various illnesses in the developed and developing countries has increased tremendously over the past decade. These remedies contain multiple active constituents which act synergistically (Peltzer, 2009). They are believed to contain pharmacological properties capable of boosting the immunity of TB-

infected individuals and aid quick recovery. The nutritive potential of a few polyherbal preparations have been investigated and found to be rich in mineral nutrients (Soni et al., 2010; Dickson et al., 2014). However, the vitamins and mineral constituents of polyherbal remedies used for the treatment of tuberculosis in Eastern Cape Province have not been investigated. Thus, this research work was designed to evaluate some important vitamins and mineral elements in polyherbal medicines used for the treatment of tuberculosis in this region.

Material and Methods

Collection of polyherbal medicines

Polyherbal medicines evaluated in this study were purchased from herbal healers in five communities namely; Alice, Fort Beaufort, Hogsback, King Williams Town and East London, all within the Amathole District Municipality, Eastern Cape Province, South Africa (Figure 8.1). The remedies were already prepared and well packaged into clean containers by the herbal healers before being transported to the Medicinal Plants and Economic Development Research Centre, University of Fort Hare, Alice, for analysis. All the herbal preparations were air dried, ground to homogeneous powder and code-named according to their respective place of collection: viz; King Williams Town site A (KWTa), King Williams Town site B (KWTb), King Williams Town site C (KWTc), Hogsback first site (HBfs), Hogsback second site (HBss), Hogsback third site (HBts), East London (EL), Alice (AL) and Fort Beaufort (FB).

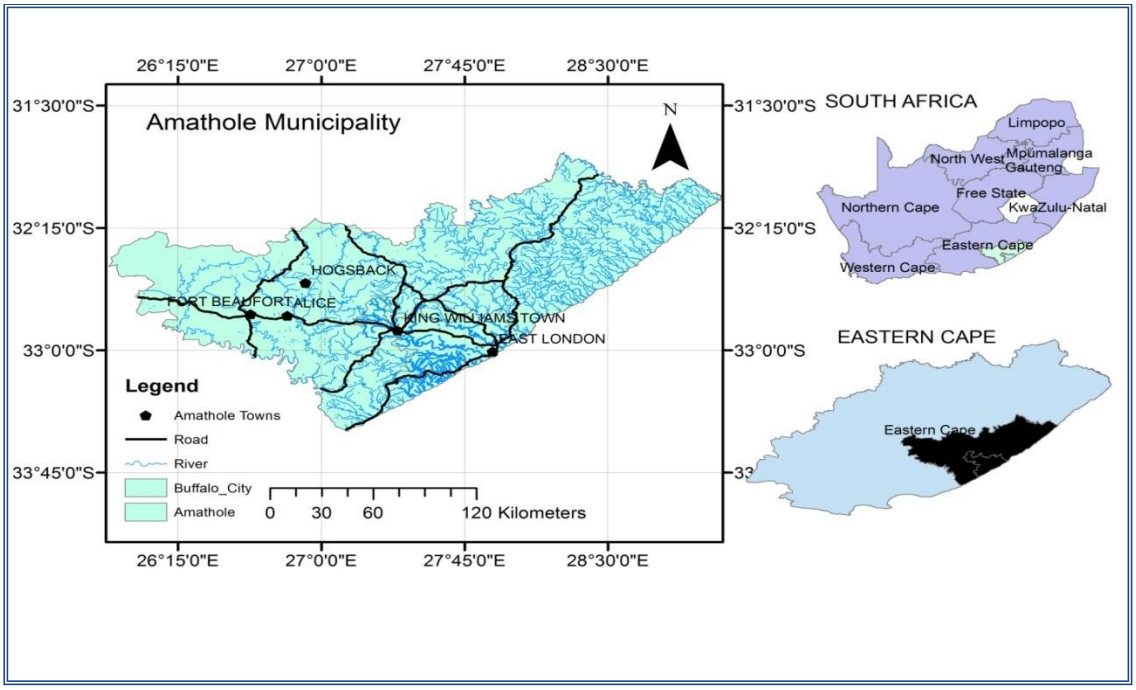

Figure 8.1: Map of Amathole District Municipality (Famewo et al., 2016)

Determination of macro and micronutrients

Digestion and analysis

The method described by Bvenura and Afolayan (2012) was used for the digestion of the dried polyherbs. Selenium powder, sulphuric acid and salicylic acid were the reagents used for digestion. The finely ground material was divided into samples of 0.3 g, which were placed in dry and clean digestion tubes. A volume of 2.5 mL of the digestion mixture was added to each tube and allowed to react at room temperature for 120 min. The tubes were heated in a block digester at 110°C for 60 min. After removal from the digester, the tubes were allowed to cool and three successive portions of 1.0 mL hydrogen peroxide were added, allowing at least 10 sec between additions because of the volatility of the reaction. The tubes were returned to the block digester at a temperature of 330°C and were removed from the block digester when the digest was colourless. The tubes were allowed to cool to room temperature before their contents were transferred to 50 mL volumetric flasks and deionized water was added to attain volumes of 50 mL. Standards were prepared for all the elements. The samples were then

analysed for the following nutrients: calcium, magnesium, sodium, potassium, nitrogen, phosphorus, iron, zinc, aluminium, manganese and copper using an inductively coupled plasma optical emission spectrometer (ICP OES; Varian 710–ES series, SMM Instruments, Cape Town, South Africa). All analyses were performed in triplicates. The macro and micro element contents were expressed as mg/100 g.

Vitamin analysis

Vitamin A content

The vitamin A (Retinol) content of each herbal remedy was determined using the method of Onyesife et al. (2014). A quantity, 1.0 g of ground sample was macerated with 20 mL of petroleum ether. This was decanted into a test tube and evaporated to dryness. About 0.2 mL of chloroform-acetic anhydride (1:1, v/v) was added to the residue. Two milliliters of TCA-chloroform (1:1 v/v) was added to the resulting solution and absorbance was measured at 620 nm using UV-3000PC spectrophotometer. Retinol standard was prepared in like manner and the absorbance was taken at 620 nm. The concentrations of standard working solutions were 10 μg/mL, 20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL and 100 μg/mL respectively. The concentration of vitamin A in the sample was extrapolated from the standard curve using the equation: $Y = 0.001x + 0.0008$.

Vitamin C content

The vitamin C (Ascorbic acid) contents of the polyherbal remedies were determined by the spectrophotometric method of Njoku et al. (2015). Briefly, 1.0 g of each remedy was macerated with 20 mL of 0.4% oxalic acid. This was filtered using Whatman No. 1 filter paper. Nine of indophenol reagent was added to 1.0 mL of the filtrate. Standard solution of vitamin C was prepared similarly and the absorbances of the standard solutions and the remedies were read at 540 nm using UV-3000PC spectrophotometer. The concentrations of

the standard working solutions were 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL and 1.0 mg/mL respectively. The concentration of vitamin C was extrapolated from the standard curve of vitamin C using the following equation:

$Y = 0.67x + 0.0824$

Vitamin E content

The vitamin E (α-tocopherol) contents of the remedies were determined using the method of Njoku et al. (2015). A quantity, 1.0 g of each remedy was macerated with 20 mL of ethanol and then filtered using Whatman No. 1 filter paper. One millilitre of 0.2% ferric chloride in ethanol and 1.0 mL of 0.5% α-α-dipyridine solution were added to 1.0 mL of the filtrate. The solution was diluted to 5.0 mL with distilled water before the absorbance was read at 520 nm using UV-3000PC spectrophotometer. The concentrations of standard working solutions ranged between 10 µg/mL and 100 µg/mL. The concentration of vitamin E in the samples was extrapolated from the standard curve using the equation: $Y = 0.0086x - 0.0216$. The vitamin contents were expressed as mg/100 g.

Statistical analysis

Each analysis was carried out in triplicates and the results were expressed as the mean ± SD. Minitab program version 17 for windows was used for the Analysis of Variance (Mintab Inc., Pennsylvania, USA). Statistical significance was evaluated at $P \leq 0.05$.

Results

Macro nutrient compositions

The macro nutrients detected in the polyherbal remedies include calcium, magnesium, sodium, potassium, nitrogen and phosphorus (Table 8.1). The results clearly indicated that these remedies are rich sources of macro nutrients. The level of potassium content was

markedly higher in EL remedy, followed by calcium and nitrogen while the least content was phosphorus. Calcium was the highest content found in KWTa and HBfs remedies. This was followed by nitrogen, potassium, sodium, magnesium and phosphorus in descending order respectively. Nitrogen was markedly higher in FB remedy while the nutrient found in the lowest level was phosphorus. KWTb and KWTc remedies contained high calcium content, followed by nitrogen and potassium with sodium being the lowest nutrient observed in the remedies. Calcium, potassium and nitrogen were the nutrients having highest values in AL, HBss, and HBts remedies respectively.

However, across the herbal formulations, calcium was the nutrient with the highest values detected, except in EL and FB remedies were potassium and nitrogen were the highest respectively. However, the lowest macro nutrients in majority of the remedies were phosphorus and magnesium in HBts remedy (Table 8.1).

Micro nutrient compositions

The micro nutrients that were determined include iron, aluminium, zinc, manganese and copper. Iron was the highest mineral nutrient detected in KWTa, AL, HBft, HBss and HBts remedies. This was followed by aluminium, manganese, zinc and copper in descending order respectively. Also, KWTc remedy had iron as the mineral with the highest micro nutrient while manganese was the highest in FB remedy. The element with the highest concentration in EL remedy was aluminium, with copper being the mineral content with the lowest value in the remedy. In KWTb remedy, aluminium was the highest mineral content, followed by iron, manganese, zinc while the lowest was copper. In general, these polyherbal remedies are good sources of iron. However, the lowest micro nutrient content in all the remedies was copper.

Vitamin compositions

Table 3 shows the vitamin contents in each of the polyherbal preparation. The vitamin A and E contents observed in the remedies vary from 95.10 to 458.20 mg/100 g and 9.00 to 9.70 mg/100 g respectively indicating that these preparations are rich sources of retinol and α-tocopherol. However, vitamin C content was not detected in all the herbal remedies.

Discussion

Polyherbal remedies are extensively used for the treatment and management of various diseases in the developing countries. According to Bhope et al. (2011) these remedies are mixtures of two or more medicinal herbs, which contain multiple active constituents and act synergistically against infections. These results have clearly indicated that the nine polyherbal remedies are rich sources of macro and micro nutrients. Calcium which was the highest mineral detected in the herbal preparations is an important mineral required in the body for the normal development and maintenance of the skeleton. It helps in the proper functioning of neuromuscular, blood clotting, nerve transmission, oocyte activation and cardiac function (Pravina et al., 2013). The mineral is stored in the teeth and bones where it provides structure and strength to the body (NHMRC, 2005; Pravina et al., 2013). Inadequate intake of this mineral could lead to rickets in children or osteoporosis in aging adults, particularly among women (IOM, 2010).

The potassium content present in the herbal remedies was between 160.00 and 2,050.00 mg/100g. The level of this mineral was markedly higher in EL remedy when compared with other polyherbal formulations. The nutrient is the major cation of intracellular fluid in the body, playing an important role in the synthesis of proteins and normal cell functioning such as digestion and neurotransmission (NHMRC, 2005). It helps to maintain the pH inside every cell and acts as an electrolyte, a molecule that transmits electrical activity between cells. It

also helps in maintaining proper nerve function and muscle contraction (Young, 2012). Thus, the presence of this mineral in the remedies could aid the normal cell functioning of the consumers considering their immunocompromised status.

In addition, the results revealed that FB and KWTc remedies are rich in nitrogen. This element is required for the growth and repair of worn-out tissues (NHMRC, 2005). Also, the polyherbal formulations are good sources of magnesium, the nutrient that acts as a cofactor to more than 300 enzyme systems which regulate diverse biochemical reactions in the body (IOM, 1997; Rude, 2010; 2012). It is involved in many physiologic pathways such as energy production, oxidative phosphorylation and glycolysis. Magnesium is required for the synthesis of nucleic acid and antioxidant glutathione, as well as contributing to the structural development of the bone. This element also plays a role in the active transport of calcium and potassium ions across cell membranes (Rude, 2012).

Sodium, which is a cation required to maintain extracellular fluid volume and serum osmolality in the body was also detected in the remedies. The nutrient plays an essential role in keeping the fluids and electrolytes in the body balance, transmit nerve impulses, helps in maintaining the membrane potential of cells and active transport of molecules across cell membranes (Capra, 2006). The sodium contents in these herbal formulations were between 6.70 and 381.00 mg/100 g. The low sodium content observed in these therapies is of great advantage because, excessive intake of sodium in human results in arterial hypertension. This mineral is required in small amount; too much intake of it has been associated with increased risk of high blood pressure leading to heart disease, stroke and kidney disease (NHMRC, 2005).

The lowest macro nutrient in the majority of the remedies was phosphorus. The main importance of this mineral is in the formation of bones and teeth. Phosphorus, which is

present in smaller amounts in cells and tissues helps to filter out waste in the kidneys. It plays an essential role in the storage and utilization of energy in the body. The element is required for the growth, maintenance and repair of all tissues and cells (IOM, 1997). Also, it helps in the production of genetic building blocks, DNA and RNA in the body. However, since calcium and phosphorus are associated with each other for growth and maintenance of bones, teeth and muscles; the presence of calcium in the polyherbal formulations can augment for the low phosphorus observed.

Also, these polyherbal remedies are good sources of iron, which is an essential component of a number of proteins including haemoglobin (Wessling-Resnick, 2014), myoglobin (Aggett *et al*., 2012), cytochromes and enzymes involved in redox reactions (NHRMC, 2005). It also plays an important role in metabolism, development, normal cellular functioning and synthesis of some hormones and connective tissues (Murray-Kolbe et al., 2010; Aggett et al., 2012). The deficiency of this nutrient could increase the susceptibility of an individual to infection such as tuberculosis (Karyadi et al., 2000). Thus, the presence of iron in the polyherbal remedies is of great importance.

Copper was the lowest mineral detected in the herbal preparations. The nutrient is one of the cofactors for antioxidant enzymes called superoxide dismutase in the body. It is required to manufacture collagen, a major structural protein in the body. It helps with incorporation of iron into red blood cells, prevents anaemia and involved in the generation of energy from carbohydrates. It is also important for nerve function, building strong tissue, bone growth, maintaining blood volume and helps the body in the utilization of sugar (IOM, 2001; NHMRC, 2005). The presences of this mineral in conjunction with other minerals detected could improve the immunity of the consumers.

Also, manganese was equally observed in the polyherbal remedies. This nutrient plays an important role in the formation of bone, metabolism of fat and carbohydrate, cholesterol and amino acids (IOM, 2001; NHMRC, 2005). It helps the body to form connective tissue, blood-clotting factors and sex hormones. Manganese, a component of the antioxidant enzyme which helps to fight free radicals also plays a role in calcium absorption and blood sugar regulation (IOM, 2001).

Zinc is another important micro nutrient playing a significant role in the immune system of an individual and helps to protect the cells against various free radicals. While some of these herbal formulations had low zinc level, three of the remedies namely; EL, AL and HBss were rich in zinc. The importance of this element cannot be ignored as it is involved in various functions such as cellular metabolism, catalytic activity of enzymes, protein synthesis, wound healing, DNA synthesis and cell division. The deficiency of Zn can leads to impairment of immunity and thus increases susceptibility to infections such as TB (Ramakrishnan et al., 2008; Muthuraj et al., 2010).

Vitamins are important in the body. They help to maintain the physiology of the body and boost the immune system. In this study, vitamin A had the highest concentrations followed by vitamin E. However, vitamin C was not detected in any of the remedies. While Vitamin A supports the vision and bone growth in the body, vitamin E strengthens the immune system and repairs the DNA (Martini et al., 2010), Vitamin E is an anti-oxidant capable of playing the role of vitamin C in cavity healing in active cavitary tuberculosis (Chakraborty et al., 2014). The supplementation of vitamin E and selenium reduces oxidative stress and enhances total antioxidant status in patients with pulmonary TB treated with standard chemotherapy (Seyedrezazadeh et al., 2008). This vitamin also helps in preventing the peroxidation of membrane phospholipids and cell membrane oxidation through its anti-oxidant properties (Njoku et al., 2015).

These vitamin and mineral constituents play important roles in the treatment of tuberculosis and also serve as cofactors for many physiologic and metabolic functions (Safarian et al., 1989). According to SANDH (2001), patients of TB requires multivitamin and mineral supplement which provides about 50 to 150% of the recommended daily allowance.

Conclusion

The findings of this study indicated that these polyherbal medicines are rich sources of vitamins and essential nutrients. Considering the amount of vitamins and mineral constituents in the polyherbal preparation, it could be deduced that these remedies are probably boosting the immune system of TB patients thereby aiding their recovery.

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Conflict of interest

The author declares no conflict of interest.

Table 8.1: Macro nutrient compositions (mg/100 g) of nine polyherbal remedies used for the treatment of tuberculosis in the Eastern Cape Province, South Africa

Sample	N	Ca	Mg	Na	K	P
EL	803.30 ± 0.08^{ac}	1956.70 ± 0.02^{ae}	406.70 ± 0.02^{a}	381.66 ± 8.20^{a}	2050 ± 0.20^{a}	263.33 ± 5.77^{a}
KWTa	626.70 ± 0.16^{bd}	2113.30 ± 0.89^{a}	160.00 ± 0.04^{b}	184.25 ± 64.58^{b}	483.33 ± 0.11^{b}	76.67 ± 25.17^{b}
AL	713.30 ± 0.03^{bc}	973.30 ± 0.02^{b}	216.67 ± 0.00^{c}	362.57 ± 5.99^{a}	806.67 ± 0.01^{c}	146.67 ± 5.77^{c}
HBfs	583.30 ± 0.05^{bd}	1376.70 ± 0.09^{bc}	80.00 ± 0.01^{d}	300.59 ± 9.07^{c}	373.33 ± 0.03^{b}	73.33 ± 15.28^{b}
HBss	590.00 ± 0.03^{bd}	1363.30± 0.11^{bc}	156.67 ± 0.01^{b}	113.25 ± 10.81^{d}	760.00 ± 0.05^{c}	123.33 ± 5.77^{c}
HBts	903.30 ± 0.07^{ae}	2180.00 ± 0.03^{a}	110.00 ± 0.00^{e}	201.57 ± 10.92^{b}	970.00 ± 0.04^{d}	143.33 ± 5.77^{c}
FB	1866.70 ± 0.08^{f}	1120.00 ± 0.02^{bc}	260.00 ± 0.00^{f}	276.27 ± 5.59^{c}	1660.00 ± 0.04^{e}	200.00 ± 10.00^{d}
KWTb	560.00 ± 0.03^{d}	1567.00 ± 0.21^{ce}	140.00 ± 0.01^{b}	20.52 ± 1.34^{e}	160.00 ± 0.03^{f}	46.67 ± 5.77^{f}
KWTc	1023.30 ± 0.08^{e}	6503.30 ± 0.07^{d}	76.67 ± 0.00^{d}	6.73 ± 0.02^{e}	200.00 ± 0.01^{f}	20.00 ± 0.00^{e}

King Williams Town site A (KWTa), King Williams Town site B (KWTb), King Williams Town site C (KWTc), Hogsback first site (HBfs), Hogsback second site (HBss), Hogsback third site (HBts), East London (EL), Alice (AL) and Fort Beaufort (FB).

Each value represents the mean ± standard deviation for triplicate determinations. Different superscripts within the same column are significantly different ($P \leq 0.05$).

Table 8.2: Micro mineral compositions (mg/100 g) of nine polyherbal remedies used for the treatment of tuberculosis in the Eastern Cape Province, South Africa

Sample	Zn	Cu	Mn	Fe	Al
EL	8.13 ± 0.65^{a}	1.31 ± 0.11^{a}	6.70 ± 1.60^{a}	112.70 ± 48.54^{a}	158.70 ± 69.57^{a}
KWTa	1.70 ± 0.56^{b}	0.61 ± 0.44^{b}	39.13 ± 14.75^{b}	54.6 ± 38.54^{b}	39.87 ± 23.09^{b}
AL	3.03 ± 0.12^{c}	0.56 ± 0.04^{b}	7.43 ± 0.15^{a}	35.57 ± 1.04^{bc}	30.50 ± 0.85^{b}
HBfs	0.90 ± 0.05^{d}	0.19 ± 0.01^{c}	8.57 ± 0.37^{a}	24.63 ± 15.91^{bc}	11.8 ± 0.70^{b}
HBss	3.03 ± 0.35^{c}	0.51 ± 0.09^{bd}	3.40 ± 0.20^{a}	18.93 ± 5.21^{bc}	13.07 ± 1.40^{b}
HBts	2.17 ± 0.12^{b}	0.56 ± 0.18^{b}	5.37 ± 0.12^{a}	20.17 ± 4.38^{bc}	17.97 ± 1.85^{b}
FB	2.17 ± 1.49^{b}	0.67 ± 0.19^{b}	18.67 ± 0.29^{c}	14.70 ± 1.18^{c}	15.07 ± 1.04^{b}
KWTb	0.70 ± 0.02^{de}	0.25 ± 0.04^{cd}	23.77 ± 2.79^{c}	32.67 ± 4.15^{bc}	45.57 ± 5.84^{b}
KWTc	0.30 ± 0.17^{e}	0.25 ± 0.03^{cd}	58.03 ± 2.49^{d}	76.50 ± 30.20^{bc}	44.30 ± 0.06^{b}

King Williams Town site A (KWTa), King Williams Town site B (KWTb), King Williams Town site C (KWTc), Hogsback first site (HBfs), Hogsback second site (HBss), Hogsback third site (HBts), East London (EL), Alice (AL) and Fort Beaufort (FB). Data expressed as mean ± SD of three replicates. Different superscripts within the same column are significantly different ($P \leq 0.05$).

Table 8.3: Vitamin contents (mg/100g) detected in nine polyherbal remedies used for the treatment of tuberculosis in the Eastern Cape Province, South Africa

Sample	Vitamin A	Vitamin E
EL	459.20 ± 0.02^{a}	9.40 ± 0.01^{ae}
KWTa	262.20 ± 0.02^{b}	9.50 ± 0.02^{ab}
AL	124.80 ± 0.02^{c}	9.0 ± 0.02^{cd}
HBfs	197.00 ± 0.00^{d}	9.50 ± 0.01^{bd}
HBss	200.80 ± 0.00^{d}	9.30 ± 0.03^{e}
HBts	333.10 ± 0.02^{e}	9.40 ± 0.01^{e}
FB	365.40 ± 0.00^{e}	9.00 ± 0.03^{f}
KWTb	201.80 ± 0.00^{d}	9.70 ± 0.05^{c}
KWTc	96.10 ± 0.01^{c}	9.30 ± 0.04^{e}

Data expressed as mean ± SD of three replicates. Different superscripts within the same column are significantly difference ($P \leq 0.05$).

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CHAPTER NINE

GENERAL DISCUSSION, CONCLUSION AND CONTRIBUTION TO KNOWLEDGE EMANATING FROM THIS STUDY

CHAPTER NINE

TABLE OF CONTENTS

Contents **Page No**

General Discussion

Tuberculosis has remained a serious threat to human health, with about one-third of the global population infected. Despite the development of anti-tubercular agents, millions of people are still suffering and dying from the disease. In 2015, about 10.4 million new cases of tuberculosis were reported worldwide, out of which 1.4 million deaths were recorded (WHO, 2016). Due to non-compliance of patients to the use of TB-drugs, poor access to TB-drugs, the rise in drug resistance, nature of the lipid-rich cell wall of TB, coupled with the association with HIV infection have worsened the global situation of tuberculosis; thus difficult to control (Kumar et al., 2017).

In the developing countries, cases of tuberculosis infection are rampant in low-income countries like South-East Asia and Africa. It was estimated that in every 100,000 people living in Sub-Saharan Africa, 350 are infected (WHO, 2016). South Africa is one of the six countries with the highest burden of tuberculosis, HIV-associated TB and the second highest number of diagnosed multidrug-resistant TB cases (Churchyard et al., 2014). With the continuing global epidemic of tuberculosis, coupled with the resistance of this organism to anti-tubercular agents, there is a need to develop new drugs from the herbal origin (Bhatcha, 2013).

The use of herbal medicines for the treatment and management of various illnesses has increased tremendously in the developed and developing countries. It was estimated that about 80% of the population in the developing countries make use of traditional medicine for their primary health care (WHO, 2002). The use of modern medicine in the treatment of complex disease such as tuberculosis is associated with the problem of cross-resistance. However, herbal drugs often produce a promising effect in this context over a single purified drug (Mehta et al., 2015; Aslam et al., 2016). Many medicinal plants have been reported in

different parts of the world to possess anti-tubercular activity. These plants often contain phytoconstituents such as alkaloids, tannins, flavonoids, xanthones, triterpenes and quinines which are involved in the anti-tubercular activity (Arya, 2011; Bhatcha, 2013). Medicinal plants used in combination as polyherbal formulations have shown to have potential interactive effects which usually produced maximum benefit and effective therapeutic purpose (Nadeem et al., 1996). Some of these effects include mutual enhancement, mutual assistance, mutual restraint and mutual antagonism (Ramaiah et al., 2013).

In South Africa, about 72% of the black African population use herbal medicines for their health needs (Mander et al., 2007). Studies have shown that the people living in the Eastern Cape Province make use of herbal medicines for the treatment of tuberculosis and associated diseases (Buwa and Afolayan, 2009; Lawal et al., 2014). However, there is no information on the documentation of the polyherbal medicines used for the treatment of tuberculosis in the Province, as well as the scientific evidence to justify the ethno-medicinal use and guarantee the safety of the consumers.

Ethno-medicinal documentation of polyherbal medicines used for the treatment of tuberculosis

The indigenous knowledge of herbal medicines in many parts of the world is fast disappearing due to the transformation of traditional culture (Hussain et al., 2010). The survey carried out in this project showed that nine polyherbal medicines made from 24 plant species, belonging to 20 families are used for the treatment of tuberculosis in the study area (Chapter 2). While the frequently mentioned plant families are Apiaceae, Liliaceae, Strychnaceae, Rutaceae and Hypoxidaceae; the four most commonly used plants are *Allium sativum* L. (Liliaceae), *Strychnos decussata* (Pappe) Gilg. (Strychnaceae), *Daucus carota* L. (Apiaceae) and *Hypoxis argentea* (Fiscand) (Hypoxidaceae). The therapeutic claims made on

most of the medicinal plants used for the preparation of these remedies are well supported by the literature, with many of the species having antimicrobial properties (Buwa and Afolayan, 2009; Bisi-Johnson et al., 2010; Green et al., 2010). The documentation of ethno-medicinal information has a significant role in illuminating folk knowledge, which facilitates the discovery of modern allopathic drugs (Flaster, 1996; Cox, 2000; Rashid and Arshad, 2002). In order to preserve the loss of traditional knowledge of polyherbal medicines, it is necessary that inventories of plants, part used, mode of preparation and the dosage used are properly documented in systematic studies. This survey helps in the conservation of indigenous knowledge through the identification of polyherbal medicines used for the treatment of tuberculosis with market potential that can generate incomes for local communities. The knowledge also provides useful leads for scientific research, especially in the development of modern allopathic drugs destined for the international markets (Ismail and Nisar, 2010).

Molecular identification of bacterial and fungal contaminants of the polyherbal medicines

In order to guarantee the safety of the consumers, it is important to identify various bacteria and fungi present in the polyherbal medicines (Chapter 3 and 4). Several investigations have shown that herbal medicines are associated with a broad variety of contaminants such as microbial agents and heavy metals (Ting et al., 2013; Noor et al., 2014; Anyanwu, 2010; Osei-Adjei et al., 2013). Both pathogenic and non-pathogenic bacteria, as well as fungi contaminants particularly moulds and yeast, were identified in the herbal preparations. Some of the identified bacteria that are clinically pathogenic to human include *Raoultella ornithinolytica, Rahnella aquatilis, Bacillus anthracis, Bacillus cereus, Salmonella enteric, Enterobacter cloacae, Klebsiella oxytoca* and *Klebsiella pneumonia*. Others such as *Enterobacter asburiae, Paenibacillus polymyxa, Pantoea rwandensis, Klebsiella variicola* and *Pseudomonas* sp. are opportunistic pathogens causing opportunistic infections in

individuals with impaired immunity. The predominant mycoflora obtained include *Alternaria*, *Candida*, *Ramularia*, *Cladosporium*, *Penicillium*, *Aspergillus* and *Malassezia*. While some of these organisms are capable of producing mycotoxins, others could cause infections in immunocompromised patients. Different factors such as temperature, humidity and extent of rainfall during pre-harvesting and post-harvesting periods, handling practices, unhygienic production conditions and storage period could have influenced the contamination of these remedies (De Freitas Araújo et al., 2012). The presence of these microbial contaminants in the polyherbal remedies is of great concern, considering the immunocompromised status of the consumers.

Antimicrobial activity of the polyherbal medicines

Tuberculosis medications have been revolutionized due to the resistant strain of *Mycobacterium tuberculosis*, thus, there is a need to develop alternative medicines using medicinal plants. Many studies have shown that medicinal plants possess antimicrobial activity (Afolayan and Meyer, 1997; Buwa and Afolayan, 2009; Askun et al., 2012). They play a significant role in the process of drug discovery and development, as many infectious diseases have been treated by them (Newman and Cragg, 2007; Mativandlela, et al., 2008; Gupta et al., 2010). The effect of the polyherbal remedies on *Mycobacterium tuberculosis* as well as on opportunistic bacterial and fungal pathogens associated with tuberculosis infection was examined (Chapter 5 and 6). Seven of the herbal preparations showed greater anti-mycobacterial activity. This is an indication that these remedies could be potential sources of new anti-tubercular agents (Chapter 5). Only three preparations showed activity against Gram positive and negative bacteria. However, KWTa remedy showed activity against *Aspergillus* spp. while KWTc and HBts had the highest activity against *Candida albicans* (Chapter 6). The capability of these remedies to inhibit the organisms is an indication that they are potential broad-spectrum antimicrobial agents.

Toxicological evaluation of the polyherbal medicines

There is a perception that herbal medicines are generally safe, bio-friendly, eco-friendly and free from side effects. However, studies have shown that some of them are known to carry toxicological properties (Hamidi et al., 2014). According to Calixto (2000), the general idea that herbal medicines are completely safe and free from side effects because they come from natural origin is false. Not all of the herbal preparations reported to be useful are harmless because they contain biologically active chemical substances. Thus, in order to ensure the safety of the users, polyherbal medicines used for the treatment of tuberculosis in the study area were screened for toxicity using the Brine shrimp toxicity assay (Chapter 7). The herbal preparations exhibited low toxicity on the hatching success of *A. salina* cysts and on the mortality of the larvae. This is an indication that the polyherbal formulations are non-toxic, thus, they could be safe for consumption. However, further *in vivo* toxicity tests are required to validate the safety of the users.

Evaluation of some important vitamins and mineral nutrients of the herbal medicines

Vitamins and mineral nutrients are essential and are needed for normal growth and maintenance of body functions. While vitamins are responsible for a spectrum of vital functions in the body, the mineral nutrients work together for tissue regeneration, cellular integrity and play important roles in the treatment of tuberculosis (Gupta et al., 2009). Herbal medicines are believed to contain pharmacological properties capable of boosting the immunity of TB-infected patients and aid quick recovery. Thus, some important vitamins and mineral nutrients in polyherbal medicines used for the treatment of tuberculosis were evaluated (Chapter 8). The remedies are rich sources of vitamins A, E and essential nutrients. It could be deduced that these remedies are probably boosting the immune system of tuberculosis patients thereby aiding their recovery.

Conclusions

This study has provided significant ethno-medicinal information on polyherbal medicines used for the treatment of tuberculosis in the Eastern Cape Province, South Africa. This information will preserve the traditional culture of the people by preventing the loss of indigenous knowledge of polyherbal preparations. The identification of bacteria and fungi in the polyherbal medicines is a great concern. Although, the majority of the microorganisms are non-pathogenic; however, they are capable of causing infections in tuberculosis patients, considering their immunocompromised status. The study suggests that government should take adequate control measures to set specific standards for quality of these medicines. The ability of the remedies to possess activity against *Mycobacterium tuberculosis* and their effect on the organisms capable of causing secondary infections in tuberculosis patients gives confidence to their therapeutic usage as anti-*Mycobacterium tuberculosis*. Also, the toxicological evaluation of the remedies indicated that they are safe for consumption. The presence of vitamins, macro and micro nutrients in the herbal preparations support their folkloric use as they can serve as dietary supplements boosting the immune system of the tuberculosis patients. The findings of this study provide scientific validation of the use of polyherbal medicines in the treatment of tuberculosis and associated diseases in the Eastern Cape Province of South Africa.

Contributions to knowledge

1. The study is the first to have documented the list of ingredients such as the name of the plants including the non-herbal inclusions, type and dosage of polyherbal formulations used for the treatment of tuberculosis in South Africa. This might help in preventing the loss of indigenous knowledge on the preparation of polyherbal medicines used for the treatment of tuberculosis.
2. The study successfully identified bacterial and fungal contaminants in the polyherbal remedies. This will help in creating awareness to the herbal healers and the consumers that proper hygienic condition is required during the preparation stages and the storage period. The study has shown that there is a need for government to take adequate control measures to set specific standards for quality of these medicines considering the immunocompromised status of the consumers.
3. The study has further provided scientific insight by showing the herbal remedies as possible anti-tubercular agents. This could also provide useful leads in the development of modern allopathic drugs that will solve the problem of multi-drug resistant tuberculosis.
4. The toxicity assay carried out has shown that the remedies are safe for consumption; however, further *in vivo* analysis is required.
5. Also, the study has further revealed the nutritive properties of the polyherbal medicines, thus, suggesting that they could probably help in boosting the immune system of tuberculosis patients thereby aiding their recovery.

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APPENDICES

Appendix **Page No**

LIST OF ABBREVIATIONS

AIDS - Acquired Immune Deficiency Syndrome

AL - Alice

CAM - Complementary and Alternative Medicine

DNA - Deoxyribonucleic Acid

EL - East London

EMB - Ethambutol

FB - Fort Beaufort

HBfs - Hogsback first site

HBss - Hogsback second site

HBts - Hogsback third site

HIV - Human Immunodeficiency Virus

INH - Isoniazid

KWTa - King Williams Town site A

KWTb - King Williams Town site B

KWTc - King Williams Town site C

MDR-TB - Multi-drug Resistant-Tuberculosis

MIC - Minimum Inhibitory Concentration

PZA - Pyrazinamide

RIF - Rifampicin

rRNA - Ribosomal ribonucleic acid

TB – Tuberculosis

TM – traditional medicine

WHO - World Health Organisation

XDR-TB - Extremely-drug Resistant-Tuberculosis

LIST OF TABLES

Table **Page No**

LIST OF FIGURES

Figures **Page No**

POLYHERBAL MEDICINES INVESTIGATED IN THIS STUDY

A: AL

B: KWTa

C: KWTb and KWTc

D: EL

E: FB

AL: Alice
KWTa: King Williams Town site A
KWTb: King Williams Town site B
KWTc: King Williams Town site C
EL: East London
FB: Fort Beaufort
HBfs: Hogsback first site
HBss Hogsback second site
HBts: Hogsback third site

F: HBfs

G: HBss

H: HBts

Pictures of the polyherbal medicines used in this study

ETHICAL CLEARANCE

University of Fort Hare
Together in Excellence

ETHICAL CLEARANCE CERTIFICATE
REC-270710-028-RA Level 01

Certificate Reference Number:	AFO061SFAM01
Project title:	**Evaluation and identification of microbial contaminants in selected herbal medicines used for the treatment of tuberculosis in Amathole District Municipality in the Eastern Province, South Africa.**
Nature of Project:	PhD in Biochemistry and Microbiology
Principal Researcher:	Elizabeth Famewo
Supervisor:	Prof A.J Afolayan
Co-supervisor:	Prof A.M Clarke

On behalf of the University of Fort Hare's Research Ethics Committee (UREC) I hereby give ethical approval in respect of the undertakings contained in the above-mentioned project and research instrument(s). Should any other instruments be used, these require separate authorization. The Researcher may therefore commence with the research as from the date of this certificate, using the reference number indicated above.

Please note that the UREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the document
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research

The Principal Researcher must report to the UREC in the prescribed format, where applicable, annually, and at the end of the project, in respect of ethical compliance.

Special conditions: Research that includes children as per the official regulations of the act must take the following into account:

Note: The UREC is aware of the provisions of s71 of the National Health Act 61 of 2003 and that matters pertaining to obtaining the Minister's consent are under discussion and remain unresolved. Nonetheless, as was decided at a meeting between the National Health Research Ethics Committee and stakeholders on 6 June 2013, university ethics committees may continue to grant ethical clearance for research involving children without the Minister's consent, provided that the prescripts of the previous rules have been met. This certificate is granted in terms of this agreement.

The UREC retains the right to

- Withdraw or amend this Ethical Clearance Certificate if
  - Any unethical principal or practices are revealed or suspected
  - Relevant information has been withheld or misrepresented
  - Regulatory changes of whatsoever nature so require
  - The conditions contained in the Certificate have not been adhered to

- Request access to any information or data at any time during the course or after completion of the project.

- In addition to the need to comply with the highest level of ethical conduct principle investigators must report back annually as an evaluation and monitoring mechanism on the progress being made by the research. Such a report must be sent to the Dean of Research's office

The Ethics Committee wished you well in your research.

Yours sincerely

Professor Wilson Akpan
Acting Dean of Research

23 November 2016

INFORMED CONSENT FOR THE HERBAL HEALERS

I have been informed that my responses recorded in the questionnaire presented, will be used for academic purposes only as captioned above.

I acknowledge that I have been given an explanation of the objectives of the study.

I understand that the interview may take up to 15 minutes maximum.

I understand that my responses will be kept anonymous.

I have the right to withdraw at any time before the interview is complete.

I acknowledge that my questions have been answered to my satisfaction.

UREC has approved the research and Sponsors of the study, study monitors or auditors or UREC members may need to inspect research records.

By my signature below, I consent to be interviewed.

Informant Signature..... Date.....

Witness/team member..... Date.....

Main purpose of the research

To collect information from the herbal healers regarding the use of polyherbal medicines used for the treatment of tuberculosis.

Objectives of the research

To investigate and document the list of ingredients such as the name of the plants used including the non-herbal inclusions, type, mode of preparation and dosage of polyherbal formulations used for the treatment of tuberculosis.